MICROBIOLOGICAL EVALUATION OF PROTEIN QUALITY

I. A COLORIMETRIC METHOD FOR THE DETERMINATION OF THE GROWTH OF TETRAHYMENA GELEII W IN PROTEIN SUSPENSIONS

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THREE FIGURES

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The nutritional quality of proteins depends, in general, on the proportion and availability of the amino acids, particularly the "essential" amino acids, which they contain. Although present analytical methods are able to approximate the amino acid content of proteins and protein-containing materials, they reveal nothing about availability. Consequently, any true assessment of the nutritional value of protein must rely on biological evaluation.

It is recognized that the biological value of a protein may vary according to the criteria, such as growth, maintenance, blood or liver regeneration, used for its measurement. Regardless of the criterion used, all methods so far proposed are time-consuming and relatively expensive. The advantages of a microbiological procedure for evaluating protein quality are obvious.

The use of *Tetrahymena geleii* H, a holotrichous ciliated protozoan, as an experimental animal in the estimation of the biological value of proteins was suggested by Dunn and Rock-

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land ('47). The growth response of this microorganism to the various proteins tested was measured by titrating the acid produced in the experimental cultures. It was later found by these workers (Rockland and Dunn, '49a) that a 41-day incubation period was necessary before maximum acid production was obtained, due, undoubtedly, to the aerobic nature of the organism.

The inconvenience of the long incubation period required for the use of the titrimetric procedure made an investigation of other methods of growth evaluation seem practical. Turbidimetric measurements of the population densities of microorganisms, as conducted by Kidder and Dewey ('48), could not be successfully employed inasmuch as media containing particulate protein would, in most instances, be opaque prior to inoculation with the organism and could not be expected to increase in density as a result of the growth of the organisms. The microscopic counting technique outlined by Johnson and Baker ('43) proved excessively tedious. For these reasons, an attempt was made to develop a rapid and simple technique for estimation of the growth of *T. geleii* in protein suspensions.

The present work describes the successful development of such a technique and indicates the applications of this method. It is based on the quantitative enzymatic reduction, by the organisms, of the colorless, water-soluble 2,3,5-triphenyltetrazolium chloride (TPTZ) to the red, water-insoluble triphenylformazan (TPF), a reaction studied extensively by Mattson et al. ('47). The TPF is dissolved in acetone and the intensity of the red solution determined colorimetrically.

EXPERIMENTAL

Technique for estimating growth of T. geleii

Organism. The organism used in these studies was the protozoan, *Tetrahymena geleii* W, obtained through the courtesy of Dr. G. W. Kidder.[2] The H strain used by Dunn and

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Rockland was not employed inasmuch as the amino acid requirements of this strain are more complex than are those of the W strain. Kidder and Dewey ('45a) have reported that the latter strain requires only the 10 amino acids "essential" to the growth of the rat, while the H strain requires serine in addition to these 10.

Stock organisms were grown in bacteria-free culture in 125 ml of 0.2% yeast extract [3] in 250-ml, cotton-plugged Erlenmeyer flasks. The cultures were incubated at 25°C. Transfers were made aseptically, using a bacteriological loop.

Reagents. The 2,3,5-triphenyltetrazolium chloride [4] was prepared in a 1% solution in pH 7.7 phosphate buffer. The buffer was prepared by diluting 50 ml of 0.1 M KH_2PO_4 plus 45.2 ml of 0.1 N NaOH to 100 ml with distilled water.

The 0.1 M $HgCl_2$ used to stop the enzymatic reaction was prepared using 20 ml of concentrated HCl per liter.

Reagent grade acetone [5] was used to dissolve the TPF formed by the organisms.

Method. The procedure for estimating the growth of *Tetrahymena* was developed using a 6-day stock culture. Duplicate levels of graded amounts of this culture were pipetted in 15-ml centrifuge tubes and 1% TPTZ, made up in pH 7.7 buffer, was added in a ratio of 1 ml to 5 ml of culture fluid. A blank solution, containing 10 ml of culture which had been autoclaved at 15 lb. pressure for 5 minutes, was similarly treated. The contents of the tubes were mixed well and the tubes were stoppered and incubated 4 hours at 25°C.

At the end of this time, acidic 0.1 M $HgCl_2$ was added to each tube in a ratio of 1 ml to 10 ml of the original culture fluid. This reagent inactivates the enzymes responsible for the reduction of the dye and thus stops the reaction without contributing any extraneous color to the solution.

The tubes were then centrifuged at 1500 r.p.m. for 10 minutes and the supernatant discarded. The tubes were allowed

[3] Bacto Difco.

[4] VitaStain, Arapahoe Chemicals, Inc., Boulder, Colorado.

[5] Baker and Adamson.

to drain in an inverted position for about 5 minutes and the excess liquid wiped off the lips of the tubes before they were set upright in the rack.

Ten milliliters of acetone were added to each tube and the suspension stirred to allow complete extraction of the TPF. The tubes were again centrifuged at 1500 r.p.m. for 10 minutes to clear the acetone solution of insoluble mercury salts. The optical density of the supernatant was then determined, using a Coleman Model 14 Spectrophotometer. Readings were

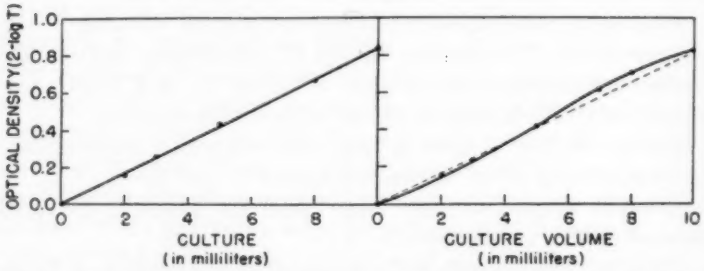

Fig. 1 Six-day *T. geleii* W used; grown in 125 ml 0.2% yeast extract in 250-ml Erlenmeyer flasks. Left-hand graph obtained with undiluted culture. Right-hand graph obtained by adjusting all culture aliquots to equivalent final volumes (10 ml) with autoclaved culture blank. Dotted line represents a straight line drawn through the points at zero and 5 ml.

made at 470 mμ, using a PC-4 filter. The instrument was adjusted to zero with the blank solution prepared from autoclaved culture.

The data obtained using this procedure are plotted in figure 1. The linear response indicates that the amount of dye reduced is proportional to the number of organisms present, and thus the method appears to be applicable to determinations of the growth response of the animal.

When this sort of determination is repeated, using blank culture to adjust the volume in all tubes to 10 ml before the addition of 2 ml of 1% dye to each tube, the curve is somewhat distorted (see fig. 1). The reason for this phenomenon is obscure. It would appear that the autoclaved culture used

as a blank has an inhibitory effect on the dye reduction by the organism.

Growth response of T. geleii to amino acids

In order further to test the validity of the method in measuring the growth response of the organism, it was decided to see whether dose-response curves of each of the 10 "essential" amino acids with the organism could be obtained using the colorimetric procedure.

The basal medium used in these studies was that of Kidder and Dewey ('49), modified for the present work by the omission of tween 85, which has been found to inhibit the growth of the organism, the substitution of the extract [6] prepared according to the method of Kidder and Dewey ('45b) for "protogen," and the inclusion of a liver fraction, the preparation of which is described by Rockland and Dunn ('49b). In addition, the yeast nucleic acid was not hydrolyzed, as suggested by these authors, but was added from a freshly-prepared solution of sodium nucleinate.[7] The basal medium for each of the growth tests included all of the amino acids except the one to be tested. In these assays the organisms were grown in 25-ml volumes in 50-ml, cotton-plugged Erlenmeyer flasks for three days at 25°C. Either 5 or 10 ml of live culture and the same amount of the corresponding blank solution were tested, using the TPTZ method. The dye used in this and subsequent work was a commercial product.[8] The use of a 1.3% solution of this preparation was necessary to produce color comparable to that observed using a 1% solution of the other TPTZ product.[9]

It can be seen from figure 2 that the graded growth of the organism in the dose-response curves is reflected by the use of the colorimetric method. These data serve to confirm the

[6] Cerophyl.

[7] Merck.

[8] It was 2,3,5-triphenyltetrazolium chloride, Schwarz Laboratories, Inc., New York, N. Y.

[9] See footnote 4, page 337.

hypothesis that this method can be used to measure the growth or population density of the organism in experimental cultures.

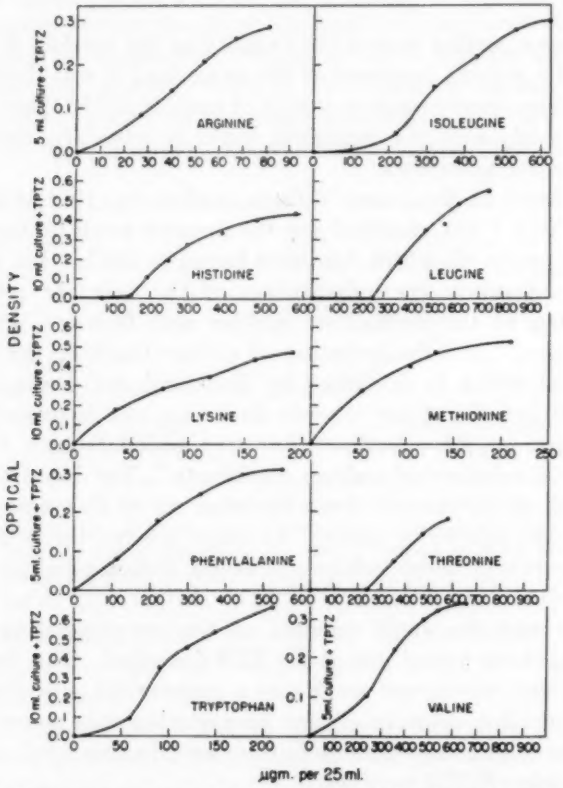

Fig. 2 Dose-response curves of the 10 "essential" amino acids with *T. geleii* W. The organism was grown in 25-ml volumes in 50-ml Erlenmeyer flasks for three days at 25°C. Basal medium was that of Kidder and Dewey ('49) as modified for the present work.

Growth response of T. geleii to proteins

Additional work was then undertaken to test the usefulness of the method in assays of protein quality in which the organ-

isms are grown in the presence of whole protein. Accordingly, animal liver meal, casein, whole egg, and soybean oil meal (extracted process) were incorporated into the basal medium from which the amino acids had been omitted. The proteins at several nitrogen levels each were first homogenized in distilled water in the Potter-Elvehjem apparatus (Potter, '48), since it was found that this treatment markedly improved the growth response of the organism, especially to the

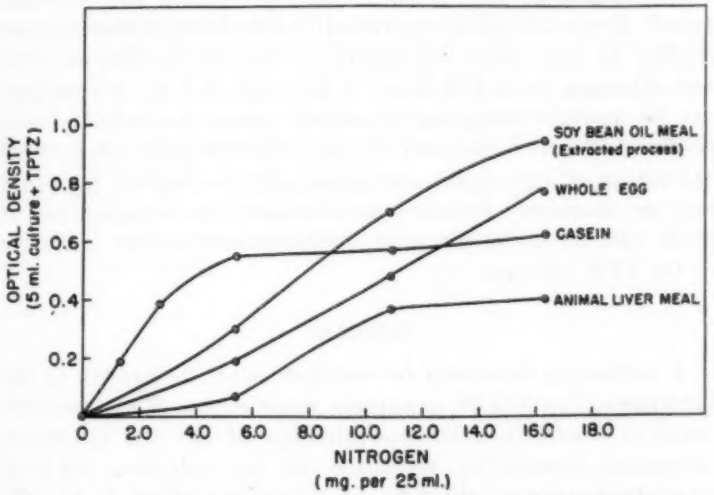

Fig. 3 *T. geleii* W cultures were grown in 25-ml volumes in 50-ml Erlenmeyer flasks for 5 days at 25°C. The basal medium was that of Kidder and Dewey ('49) as modified for the present work, and the respective proteins were substituted for the amino acids.

crude protein in animal liver meal. The homogenates were then supplemented with the basal medium and 25-ml aliquots of the well-mixed suspensions were placed in each of three 50-ml Erlenmeyer flasks, which were plugged with cotton and sterilized by autoclaving at 15 lb. for 15 minutes. These media were cooled and inoculated, each with one bacteriological loopful of a 5- to 8-day stock culture of *T. geleii* W, and were incubated for 5 days at 25°C.

At the end of this time, one flask of each series was autoclaved (until the steam temperature reached 115°C.) to be used for a blank solution. The culture fluid from the remaining two flasks was pipetted in duplicate in 5-ml volumes into 15-ml centrifuge tubes and subjected, along with 5 ml of the blank solution, to the TPTZ procedure as outlined previously. The optical densities of the TPF produced by each of these cultures, as plotted in figure 3, show that the growth response of the organism differs markedly for each of the proteins tested. From this pilot experiment it may be seen that further studies on the interrelationship of culture incubation time and nitrogen level will have to be made before the method can be used in assigning biological values to unhydrolyzed proteins. It may be pointed out, also, that the previous acetone extraction of any feeds containing acetone-soluble pigments may be necessary in order to eliminate the interference of these extraneous colors with measurement of the intensity of the TPF solution.

SUMMARY

A method is described for estimation of the growth of the protozoan, *T. geleii* W, in protein suspensions. The procedure involves a colorimetric determination of the red triphenylformazan formed by reduction of the colorless 2,3,5-triphenyltetrazolium chloride, a reaction effected by the respiratory enzymes of the organism.

This method has been used in obtaining dose-response curves of the organism to the 10 "essential" amino acids — arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The procedure has also been successful in reflecting the response of *T. geleii* to graded levels of 4 different proteins tested.

ACKNOWLEDGMENTS

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EDITORIAL REVIEW

ON THE RELATION OF DIETS TO THE DEVELOPMENT, PREVENTION AND TREATMENT OF CANCER, WITH SPECIAL REFERENCE TO CANCER OF THE STOMACH AND LIVER *

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FIVE FIGURES

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More and more evidence is accumulating to indicate that, at least in some animals, there is a close relationship between particular dietary factors and certain types of cancer. Cancer of the stomach and liver apparently may belong in this class.

Cancer of the stomach in man is one of the most common of all tumors. Gastric cancers are frequent in Japan and Korea (about 45% of all cancers) as in Europe and America, but are very scarce in China (about 10% or less). In spite of the racial affinity of Chinese to Japanese, the food and other habits of life of the Chinese are considerably different from those of the Japanese. It has been long suspected that gastric malignancy may be caused by chronic irritation plus a faulty diet. Careful laboratory tests have substantiated these beliefs by means of animal experimentation. Thus Fibiger in 1913 succeeded in producing lesions in the forestomach of rats, which he believed to be squamous cell car-

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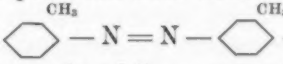
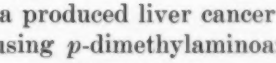
cinoma, after infesting them with the parasite *Gongylonema neoplasticum*. He thought that the tumors were elicited by the parasites, but in a paper published in 1920 he emphasized the fact that the animals were kept on a diet consisting only of white bread and water. Passey ('34, '35) suggested that such a diet must have been deficient in vitamins, and on the basis of this assumption carried out an experiment in which he showed that vitamin A deficiency, in the absence of the *Gongylonema*, led to papillomatosis of the forestomach of the rat. Stimulated by Fibiger's observations, many workers have made attempts to produce gastric cancer in different species of animals by feeding parasites, artificial and deficient diets, and chemicals known or suspected to be carcinogenic. Sixty-six such papers were written from 1918 to 1942, and of these only 17 reported production of malignant tumors of the stomach (Sugiura, '42a; Kirby, '43, '44, '45). The most interesting and most firmly established observations revealed by all these data are that the incidence of gastric lesions is definitely related to dietary deficiency and that the pathology is generally found in the rumen (forestomach).

The difference in the incidence of cancer among peoples of the East and of the West should be noted. It is a well-established fact that the incidence of primary cancer of the liver among Orientals is very much higher than the incidence of this tumor among Europeans and Americans. A statistical study on the incidence of primary liver cancer among Japanese, Chinese, Koreans and Filipinos reveals that it comprises from 6 to 10% of all cancers (Nagayo, '33). On the other hand, the incidence of primary hepatic cancers in this country, as well as in Europe, is less than 0.2% (Pack and Le Fevre, '30).

However, it is not only the racial characteristics but also the dietary habits of the East and West which differ very greatly. It has long been suspected that incidence of liver cancer is increased by chronic intoxication and unbalanced diets, and though many attempts have been made to prove that diet has some effect on the incidence of cancer, it is

only in recent years that the nature of the food consumed has been shown to exert definite influence in carcinogenesis.

In 1935 Sasaki and Yoshida first produced liver cancer in rats by feeding *o*-aminoazotoluene,  for 300 days. In 1937 Kinosita produced liver cancer in a much shorter time (150 days) using *p*-dimethylaminoazobenzene,  This compound is commercially known as Butter Yellow.[1] In both cases, rice was used as the basal food for the animals, to correspond to the staple food of the rice-eating countries of the Orient. The use of rice in these experiments is very important because, when wheat bread was given, the production of liver cancer was markedly reduced (Kinosita, '39; Okada, '40). Later on, Ando ('38) and Nakahara et al. ('38) observed that rats fed Butter Yellow did not develop liver cancer if yeast or beef liver was added to the rice diet. These classic findings of the Japanese have been confirmed by investigators at the Memorial Hospital, New York City (Sugiura and Rhoads, '41) and in other laboratories in this country.

The study at the Memorial Hospital was extended to see, first, what factor in rice was lacking and was supplied by yeast or liver and, second, to investigate the mechanism of the formation of liver tumors by the azo dyes.

As the first step, *p*-dimethylaminoazobenzene was fed (20 ml of 3% solution in olive oil to 1,000 gm of diet) to rats in a diet of unpolished rice[2] supplemented with carrots. As a result, the usual sequence began with the formation of cirrhosis, which was generally manifest at the end of 60 days

[1] Butter Yellow has been used to color oils, oleomargarine, and other vegetable fat substitutes for butter. In recent years the use of this compound in foods has been prohibited in this country. Two other yellow dyes used for butter and oleomargarine, Yellow AB and Yellow OB, are permitted. These azo dyes have been tested and found to be harmless (Sugiura, '42b, '46).

[2] It is important to use the proper kind of rice in these dietary experiments. Coarsely ground unpolished (brown) Texas or Louisiana rice is the best. Rancid, stale, or moldy rice may prevent tumor development to some extent, and therefore should not be used.

(fig. 1). As a rule, by 90 days most of the livers of the test animals show cholangiomas (bile duct tumors), and at this time some hepatomas (liver cell tumors) are also present. Then, as time goes on, the production of hepatomas increases. Ordinarily, by the 150th day the livers of all rats are the sites of a cancerous process (involving both cholangiomas and hepatomas).[3]

When young adult rats were maintained upon the Butter Yellow-rice diet without carrots, their livers were small and showed numerous small tumorous nodules and a few large ones. Histological examination revealed that about 75% of these cancers were cholangiomatous in type. When the basal diet was supplemented with carrots, the animals lived longer. The livers grew larger and showed many large cancer nodules, of both hepatoma and cholangioma types. In either case metastases in the mesentery, omentum, diaphragm and lungs were found frequently among rats living over 150 days. The induced liver tumors were transplantable by subcutaneous inoculation. The addition of 18% casein to the Butter Yellow-rice diet had a slight inhibiting effect upon the production of liver cancer. Animals on the protein sufficient diet (casein) lived much longer than those maintained on a similar diet without an extra supply of protein. The livers of these animals grew larger and showed many large cancer nodules, mostly of the hepatoma type. Cirrhosis generally did not develop. Animals on the Butter Yellow-normal diet[4] lived

[3] Subcutaneous injections of 1 ml of the 3% Butter Yellow solution in olive oil in the back once weekly for three months or more caused the formation of cirrhosis and liver tumors but much less frequently than in those animals given Butter Yellow by mouth.

[4] In the course of the investigation a study was made on the influence of a normal diet on the production of liver tumors by Butter Yellow. Ground Purina Laboratory Chow was mixed well with Butter Yellow, as in the case of the Butter Yellow-rice diet, and fed to 50 rats. The results obtained from 46 rats which lived from 100 to 300 days are as follows: 36 rats, or 78%, showed liver cancers; and 10, or 22%, had no liver tumors. In other words, Purina Laboratory Chow had only a slight inhibitory effect upon the production of liver cancer but it took longer to develop than when animals were maintained on a rice diet.

much longer than those on the Butter Yellow-rice diet, and this allowed the cancer nodules to reach an extremely large size, the liver with tumor nodules weighing from 24 to 75 gm. Livers of normal rats have an average weight of 8 gm. On the basis of these observations we are inclined to believe that the state of nutrition of the animal has much to do with the type of tumor which develops. Opie ('44) has also found that

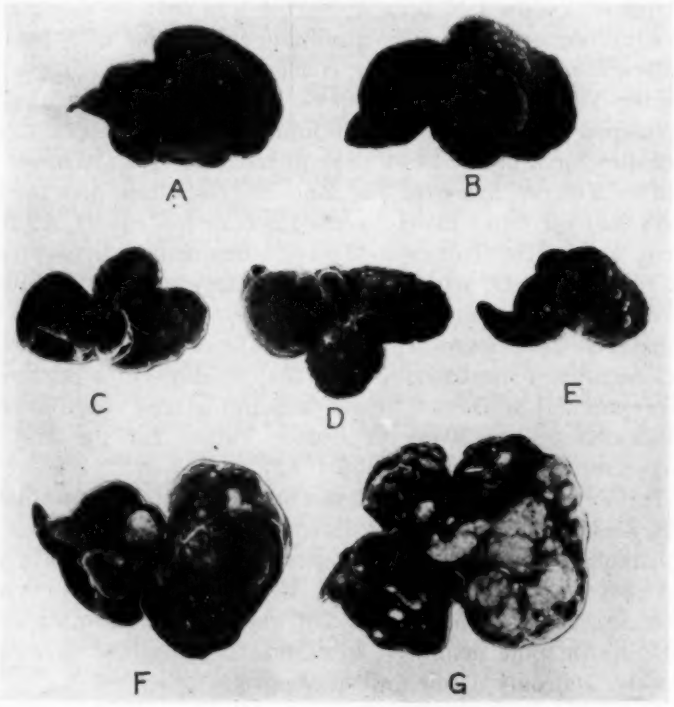


Fig. 1 Gross appearance of livers of rats fed Butter Yellow-rice diet for various lengths of time. All photographs of the livers were made at the same magnification.

A. Liver of a rat (30 days). Practically normal gross appearance.

B, C, D, E. Livers of rats (60 days). Early cirrhosis to nodular cirrhosis.

F, G. Livers of rats (120 days). Hepatomas, either isolated or very numerous.

diet determines the type of tumor. In undernourished rats the livers were abnormally small and more cirrhotic, while in well-nourished rats the livers were normal in size or even very large, with large cancer nodules and no cirrhosis.

In order to study the progress or regression of the lesions of the liver, the Butter Yellow was withdrawn from the rice diet after a definite period of ingestion. The results showed that no cirrhosis or cancer developed in the livers of any rats if Butter Yellow had been given for less than 30 days, and in very few even after the preliminary feeding with Butter Yellow for 45 days. However, if the preliminary feeding with Butter Yellow exceeded 60 days, liver cancer subsequently developed in about 80% of the animals surviving on a basal rice diet for a period of 60 days or more after withdrawal of Butter Yellow. Rats fed the Butter Yellow-rice diet for 60 days had cirrhotic livers in the majority of cases. At this time, 20% of the livers also showed adenomatous hyperplasia of the bile ducts, with beginning malignant transformation into cholangioma or hepatoma. It seems that the origin of cancer, or the changes which lead to the cancerous anaplasia, can occur in a short period (less than 60 days) if a relatively large amount of Butter Yellow is administered (each animal consumed about 250 mg of Butter Yellow for the first 60 days; see Sugiura and Rhoads, '42).

It is evident that once cirrhosis or adenomatous hyperplasia has been established in the liver of the rat it is difficult to reestablish a normal physiological state when the rats are maintained on a rice-carrot diet. Rice is poor in vitamins and protein. The rats on the rice diet were able to maintain their weights in some instances, in others they declined in weight slowly, and only a few gained slightly.

It seems that a certain factor or factors of the diet may control the experimental development of liver cancer. As mentioned before, rice is poor in vitamins and protein. The riboflavin content of brown rice was 0.5 μg/gm, while that of brewers' yeast was 70 μg/gm. The protein content of rice was 8%, while that of yeast was 49%. Three groups of 30 rats

each were maintained on a basal diet of unpolished rice mixed with 3, 6 and 15% of brewers' yeast, respectively. A control group of 50 rats was maintained on unpolished rice alone. All animals received 6 mg *p*-dimethylaminoazobenzene per 10 gm diet. All 4 groups of rats received a small amount of fresh carrots daily. The results showed that the addition of 15% of yeast to unpolished rice had a distinct protective effect against the pathological changes leading to the development of liver cancer (Sugiura and Rhoads, '41). All of the rats receiving yeast, examined between 100 and 200 days after the beginning of the experiment, had smooth and practically normal livers, grossly and microscopically (fig. 2). On the other hand, all of the rats fed the Butter Yellow-rice diet without the addition of yeast and examined on the 150th day, showed typical liver cancer. However, the inhibitory effect of yeast feeding upon the production of liver cancer was distinctly diminished when the basal diet of Butter Yellow and rice contained less than 15% yeast. With the addition of 6% yeast to the Butter Yellow-rice diet, 40% of the animals had normal livers; 30% cirrhotic livers; and 30% livers with a few tumor nodules when examined on the 150th day. With the addition of 3% yeast to the Butter Yellow-rice diet, 30% showed no malignant changes in the livers and 70% had livers with many large and small cancer nodules (fig. 3).

A similar experiment was made with dried beef liver and the results were found to be in general agreement with the results with yeast (a total of 90 rats was used). The addition of 10% liver to the Butter Yellow-rice diet had a distinct protective effect. On the other hand, the addition of 2% liver had practically no inhibitory effect upon the production of liver cancer.

It is very clear that both yeast and dried liver (beef) contain a substance or substances which are inhibitory to the production of liver cancer by feeding Butter Yellow. Yeast and liver are rich in both protein and the vitamins of the B complex. The question then arose: Is the protective effect of the yeast and liver due to the correction of a specific deficiency

or deficiencies, probaby of vitamins? In an attempt to answer this question, the effect of a vitamin or a combination of several of the B complex vitamins, with or without the addition of casein, was investigated. Studies at Memorial Hospital (Kensler et al., '41) revealed that the daily oral ad-

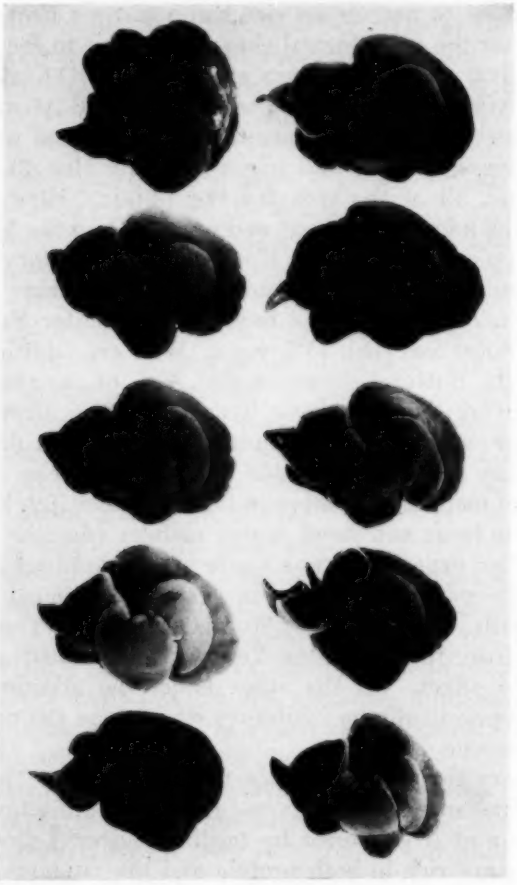


Fig. 2 Production of liver cancer in rats by Butter Yellow has been prevented by the addition of 15% yeast to the rice diet.

ministration of 20 μg of riboflavin (vitamin B_2) and 2 gm of casein, which were sufficient to promote normal growth in young rats, was almost without protective effect. However, when the daily consumption of riboflavin was increased to

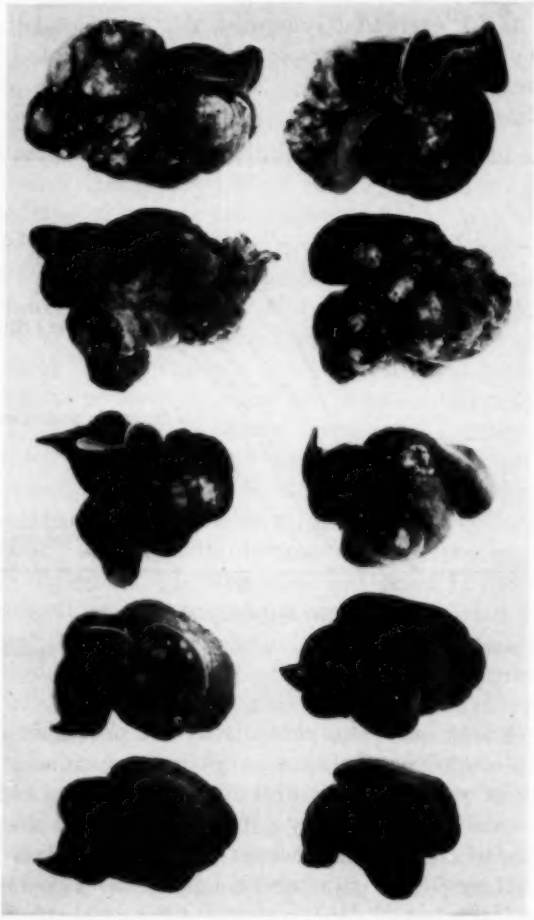


Fig. 3 The addition of 3% yeast to the Butter Yellow-rice diet had only slight inhibiting effect upon the production of liver cancer.

200 μg, the protective effect was striking, though not complete (fig. 4). From a cancer incidence of 70 to 80%, when either riboflavin or casein alone was employed, the figure decreased to an average of 3%. These findings were confirmed by Antopol and Unna ('42). Workers at Wisconsin (Miller et al., '41) and Cleveland (György et al., '41) found that other compounds, either members of or closely associated with the B complex group of vitamins, or the combination of cystine and choline, exerted a similar protective effect.

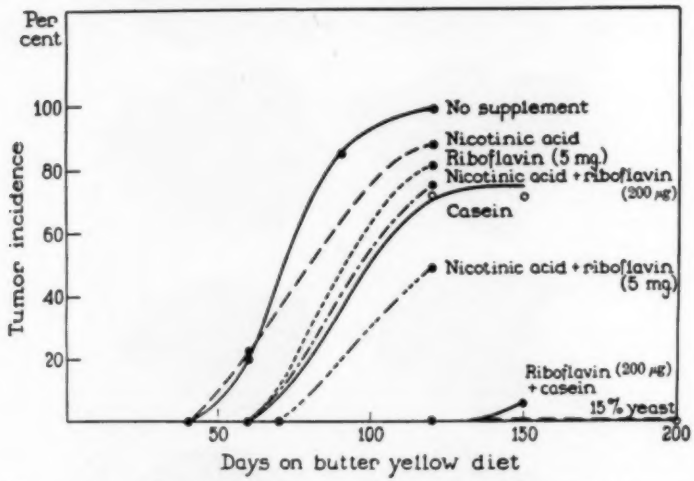


Fig. 4 Protective effect of various dietary supplements against the carcinogenic effect of Butter Yellow.

Experiments have also shown that the production of liver cancer by *o*-aminoazotoluene or *p*-dimethylaminoazobenzene is definitely reduced by the oral administration of other foodstuffs; namely, dried kidney (Mori, '41) dried spleen (Sugiura, '45; Goldfeder, '46), wheat (Ando, '40), rye (Maisin, '40), millet seed (Morigami and Kasiwabara, '41), milk (Sugiura, '44; Hoch-Ligeti, '46), water and the alcohol-soluble portions of whole beef liver (Miller et al., '41; Sugiura et al., '43), and yeast (Sugiura et al., '43), and hydrogenated coco-

nut oil (Miller et al., '44). However, the degree of this inhibition is not as striking as in the case of a combination of riboflavin and casein (Kensler et al., '41; Antopol and Unna, '42; Miner et al., '43), or a diet containing brewers' yeast or dried beef liver.

In the hope of obtaining still more complete protection, animals were fed biotin ("vitamin H") in addition to the brown rice-carrot diet or rice-casein-riboflavin diet. In contrast to results obtained with the other members of B complex groups, the addition of biotin to the diet, which was protective against the induced liver cancer, caused an increase in the incidence of tumors (du Vigneaud et al., '42). In other words, biotin was found to break down the protection. Later on it was found that rats fed Butter Yellow did not develop liver cancer if egg white or avidin was ingested at the same time (Burk et al., '43; Kline et al., '45; Harris et al., '46). This indicates that biotin can exert a procarcinogenic effect, whereas avidin possesses an anticarcinogenic effect (Kensler et al., '43). In addition to biotin, there are a number of substances which stimulate tumor formation in liver by Butter Yellow; namely, a rice bran concentrate [5] (Miner et al., '43), fat (Opie, '44), oils such as corn oil, commercial hydrogenated cottonseed oil [6] and so forth (Kinosita, '37; Miller et al., '44).

Another important question to be answered is this: Is the protection against liver cancer formation by yeast or liver feeding temporary or permanent? In an attempt to answer this question, a large number of rats were maintained upon a Butter Yellow-rice diet containing 15% yeast until the animals succumbed. The results obtained from this study may be briefly summarized as follows: of the animals which died between 150 and 250 days after the beginning of the experiment all, or 100%, had grossly and histologically normal livers; of the animals which died between 280 and 350 days, 43% had normal livers, 7% had cirrhotic livers, and 50% had livers with cancer nodules; of the animals which died between

[5] Vitap.
[6] Crisco.

400 and 700 days, 25% had normal livers and 75% had liver cancers; and of the animals which were sacrificed at 800 days, 15% had normal livers and 85% liver cancers. This indicates that only a small percentage of the animals appeared to be permanently protected by the yeast feeding.

Next, our study was directed to find whether or not primary tumors of the liver, hepatoma and cholangioma, can be treated successfully with the anticarcinogenic substances mentioned above. As in previous experiments, rats were maintained on a Butter Yellow-rice diet for definite periods of time. Then the carcinogen was removed from the food and feeding was continued with the basal rice diet with a 15% supplement of brewers' yeast or dried whole milk until the animals either succumbed or were sacrificed. The results showed that yeast or milk feeding produced a striking inhibition of liver cancer development. In rats which received the preliminary feeding with Butter Yellow for 60 days, the livers had irregular surfaces with more or less well-defined nodules. Histologically there was extensive proliferation of the fibrous connective tissue with lymphatic infiltration, with isolated islands of hyperplastic liver cells persisting and presenting the typical picture of cirrhosis. When this preliminary feeding was followed by a rice-yeast diet without Butter Yellow for 100 to 240 days, 25% had liver cancer while 75% were found to be without cirrhosis or cancer. When the Butter Yellow-rice diet was followed by a rice-milk diet without Butter Yellow for 113 to 168 days, 10% had liver cancer while 90% had smooth, practically normal livers. In contrast, 85% of the animals which were fed the Butter Yellow-rice diet for the same period, followed by rice alone, had liver cancers (fig. 5). The preceding experiments were repeated recently. As before, rats were maintained on a Butter Yellow-rice diet for 60 days, then the carcinogen was removed from the food and feeding was continued with the basal diet of brown rice, alone or mixed with 15% of yeast or dried milk, for 250 days. All of the three groups of rats received a small amount of fresh carrots daily. Rats dying before the period of 100 days are

not included in the results. These showed that of the rats kept on the rice diet, 22 out of 25 developed liver tumors. However, only 4 out of 25 rats whose diet was supplemented with yeast, and 8 out of 25 supplemented with dried milk, developed liver tumors. If the preliminary feeding with Butter Yellow exceeded 85 days, liver cancer resulted in 100% of the cases, even though the dietary anticarcinogenic yeast or liver or milk was fed for long periods of time subsequent to the Butter Yellow ingestion. Thus, liver cirrhosis produced by *p*-dimethylaminoazobenzene has been treated successfully by a rice diet containing 15% brewers' yeast, liver,

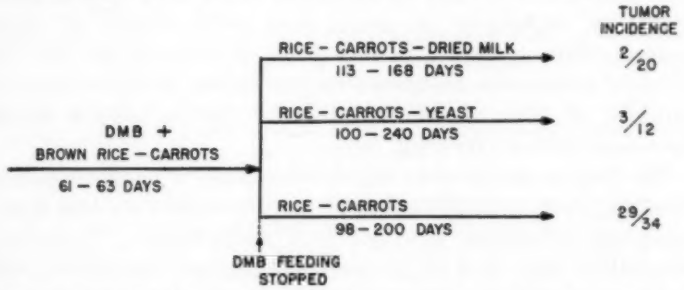


Fig. 5 Protective influence of diet on liver tumor formation after *p*-dimethylaminoazobenzene (DMB) administration is stopped.

or dried whole milk. However, once adenomatous hyperplasia of the bile ducts, cholangioma, or hepatoma had been established in the liver, these benign and malignant tumors could not be destroyed by ingestion of yeast, liver or milk. Aspiration biopsies on the livers, or laparotomies, were performed to establish the presence of liver cirrhosis and tumors.

SUMMARY

These experimental facts demonstrate that the individual components in a diet may affect the development of liver cancer in the experimental animal. Additions of some of the essential food elements to a diet will prevent tumor growth, whereas other related materials may stimulate it. Likewise,

the removal of certain elements from a diet will have a similar effect.

The unfavorable influence of Butter Yellow in producing liver cirrhosis has been counteracted by a rice diet containing 15% brewers' yeast, or liver or dried whole milk. However, once a cancerous condition has been established in the liver it is impossible to re-establish a normal physiological state by means of a diet which would prevent or greatly delay the formation of liver cancer.

These dietary influences may prove to play a very large part in the causation, prevention and treatment of human cancer. Further research along these lines may prove quite profitable, especially in conjunction with studies of other factors. For example, it would be of interest to test the effect of estrogenic hormones in preventing liver cirrhosis in animals, in view of the fact that estrogens have a strong lipotropic action (György, '48).

The factors involved in the development of neoplastic cells, resulting from a combination of dietary deficiency and a carcinogenic substance, are not well understood. There is a possibility that lack of an essential dietary substance may serve as a stimulus to the development of a mutant form of cell with alternate metabolic pathways. Data presented in this paper indicate that the action of Butter Yellow in inducing liver tumors is enhanced by dietary deficiency.

Thus, because this biological system is one in which tumor growth can be partially controlled, and because the system offers normal as well as tumor tissue for comparison, further investigation on a biological level, particularly enzyme studies of the tissue and blood, is indicated. Through this type of approach an understanding of the factors determining tumor growth should be gained.

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GROWTH-PROMOTING ACTIVITY OF BOUND PANTOTHENIC ACID IN THE RAT [1]

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The existence of several different bound forms of pantothenic acid in nature (Neal and Strong, '43; Lipmann et al., '47; King et al., '49; Brown et al., '50) raises the question of their nutritional value in higher animals. Hegsted and Lipmann ('48) have reported that preparations of coenzyme A promoted the growth of chicks at rates corresponding to their contents of pantothenic acid when the coenzyme was injected, but that when fed, coenzyme A was only about 60% as active as free pantothenic acid. A pantothenic acid conjugate has recently been found active as a source of pantothenic acid for rats in this laboratory (King et al., '50) and McRorie et al. ('50) have reported that a *Lactobacillus bulgaricus* factor (LBF) concentrate can replace pantothenic acid for rats. In the present study appropriate fractions of yeast and liver have been assayed for pantothenic acid by microbiological procedures and also by a rat growth method. Comparative studies were also made with panthenol (Jürgens and Pfaltz, '44) and with synthetic mono- and diphosphates of pantothenic acid.

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METHODS

Free and total pantothenic acid were determined by established microbiological procedures (Neilands and Strong, '48; Neilands et al., '50). Coenzyme A was determined by sulfanilamide acetylation (Kaplan and Lipmann, '48), except that the results were expressed in terms of coenzyme A-active pantothenic acid by comparison with a standard CoA concentrate as previously described (Handschumacher et al., '51). For the rat assays weanling males of the Holtzman strain were fed a diet of the following percentage composition: casein 24, sucrose 64, salt mixture [3] 4, hydrogenated vegetable oil [4] containing 0.2% haliver oil 8, and the following vitamins in milligrams per kilogram of ration: choline chloride 1,000, thiamine 2, riboflavin 5, pyridoxine 2, *p*-aminobenzoic acid 5, niacin 20, inositol 2,000, biotin 0.1, and folic acid 0.2. The various supplements were incorporated into this basal ration at the expense of the sucrose. The rations were stored in a refrigerator and were made up at intervals of two to three weeks.

Animals sufficient for the assay of 6 to 8 samples were placed on screens in individual cages and were given the unsupplemented basal ration and water ad libitum for a two-week preliminary period. At the end of this time the heaviest and lightest were discarded, and the middle 90% divided into uniform groups of 6. One such group was allotted for each sample to be assayed and 4 were reserved for determination of the standard response to pantothenic acid. The experimental diets were fed ad libitum for 5 weeks and growth responses noted. Table 1 gives the results of a typical assay series.

As a representative material high in bound pantothenic acid, a commercial liver preparation [5] was fed in each series except the 4th. Other materials tested are listed in table 2.

[3] Wesson's.

[4] Crisco.

[5] Wilson's 1: 20 liver powder, kindly supplied by Wilson and Company, Chicago.

The yeast assayed was a commercial brewers' yeast.[6] For the preparation of "yeast extract" 400 gm of this yeast were stirred into 1,800 ml of hot water (about 80°C.) and the mixture was brought to boiling in a preheated glycerine bath and maintained at that temperature for 20 minutes. The mixture was allowed to stand in a cool room overnight, made up to 2,000 ml, and the cells removed by centrifuging. One liter of the supernatant was then concentrated *in vacuo* at temperatures below 50°C. to 100 gm of a thick paste.

TABLE 1

Average weights in grams of rats in a typical assay for pantothenic acid (PA)[1]

	WEEKS ON EXPERIMENTAL DIET					
	0	1	2	3	4	5
Negative control	79	94	103	110	113	115
4 μg PA per gram diet	79	100	127	150	178	185
8 μg PA per gram diet	79	116	164	200	234	255
16 μg PA per gram diet	78	130	177	211	249	275
5.6 μg PA-mono-PO_4[2] per gram diet	79	101	128	146	169	182
20 mg yeast extract per gram diet	79	111	159	196	235	239
85 mg wheat bran per gram diet	79	110	136	158	182	193
25 mg yeast per gram diet	79	111	140	175	209	221
12 mg liver powder (1: 20) per gram diet	79	111	147	187	225	247
0.09 ml acetone supernatant per gram diet	81	117	154	190	223	
LBF[3]	81	115	156	192	225	

[1] Assay series number 3. Each value represents the average weight of a group of 6 rats.

[2] PA-mono-PO_4: pantothenic acid "γ" monophosphate (King and Strong, '50) tested as the free acid.

[3] LBF: *Lactobacillus bulgaricus* factor concentrate from natural sources (Brown et al., '50); 189 units (equivalent to 6.3 μg of pantothenic acid) per gram diet.

The concentrate of the *L. bulgaricus* factor (LBF) was supplied by Snell and co-workers (Brown et al., '50; Snell et al., '50). Pantothenic acid "γ" monophosphate and the diphosphate were synthesized by the methods of King and Strong ('50). Panthenol was supplied by a commercial firm.[7]

[6] Anheuser-Busch, Inc., St. Louis: Strain G, primary dried yeast (*Saccharomyces sicoum* U.S.P.).

[7] Hoffmann-LaRoche Co.

TABLE 2

Pantothenic acid activity of various materials as determined microbiologically and by rat assay

		MICROBIOLOGICAL ASSAY		COENZYME A ACTIVITY IN TERMS OF PA [2]	RAT ASSAY [3]	RAT ASSAY / MICROBIOLOGICAL ASSAY
		Free PA [1]	Total PA			
		μg/gm	*μg/gm*	*μg/gm*	*μg/gm*	
Crude materials						
1: 20 liver powder	(1) [4]	164	366	10	550	1.50
	(2)	164	366	10	650	1.78
	(3)	164	366	10	594	1.62
	(5)	164	366	10	654	1.79
Yeast	(1)	19	183	43	265	1.45
	(3)	19	183	43	239	1.31
Wheat bran	(3)	23	30	0	51	1.70
	(4)	23	30	0	45	1.50
Rice bran extract	(1)	124	184	0	210	1.14
Bound PA concentrates						
Yeast extract	(3)	123	353		400	1.13
	(4)	123	353		405	1.15
Liver filtrate	(4)	138	502	trace	558	1.11
Liver residue	(4)	0	59		61	1.04
Acetone supernatant "a"	(3)	20	80	trace	79	1.00
Acetone supernatant "b"	(4)	33	780	trace	833	1.07
LBF concentrate	(3)	22	504	ca. 25 [5]	572	1.14
CoA concentrates						
Sample 1	(5)	0	15,000	15,000	15,920	1.06
2	(2)	0	14,000	14,000	15,500	1.11
3	(5)	0	13,000	14,100	22,000	1.69
	(6)	0	13,000	14,100	19,200	1.47
Synthetic products [6]						
PA-"γ"-PO_4 [7]	(3)	0	0.99	0	0.86	0.87
	(4)	0	1.05	0	1.02	0.97
PA-di-PO_4 [8]	(2)	0	1.10	0	1.11	1.01
	(4)	0	0.96	0	0.86	0.90
	(5)	0	0.96	0	0.94	0.98
Panthenol	(2)	0	0	0	1.04	

[1] PA: *d*-pantothenic acid.

[2] As obtained by comparison with a standard CoA concentrate (Handschumacher et al., '51).

[3] Based on the average of weight gains after three and 4 weeks.

[4] Numbers in parentheses refer to the rat assay series.

[5] Apparent activity probably due to resynthesis of CoA from LBF.

[6] PA values expressed as moles of PA activity found per mole of compound tested.

[7] PA-"γ"-PO_4: *d*-pantothenic acid "γ" monophosphate, free acid.

[8] PA-di-PO_4: *d*-pantothenic acid diphosphate, tested as the tetra sodium salt of the methyl ester (King and Strong, '50), in series 2 and as the penta sodium salt of the free acid in series 4 and 5.

The coenzyme A preparations tested were concentrates obtained from liver by an improved procedure adapted to large scale operations.[8] The liver filtrate, liver residue, and acetone supernatant (table 2) were intermediate fractions encountered during this procedure.

RESULTS

The rats on the diet deficient in pantothenic acid grew an average of 11.5 gm per week during the first two weeks; thereafter growth was progressively less, and during the 5th week it averaged only 0.5 gm for the 5 series of experiments. Some individuals failed to gain at all during the last week. Symptoms of deficiency were relatively mild, although in some series deficient animals showed "bloody whiskers" after the 4th week. Only two of 24 negative control rats died during the study; none of the supplemented rats died.

The addition of 4, 8, or 16 μg of pantothenic acid per gram of diet resulted in progressive increments of growth (table 1); on the 16 μg level the rats gained an average of 39.4 gm per week. Although the materials being assayed were usually fed at levels that supplied 6 to 8 μg of pantothenic acid per gram of diet, as determined microbiologically, the growth of the rats on these levels varied considerably with the material fed (tables 1 and 2). One group of crude natural materials stimulated a more rapid rate of growth than that expected on the basis of the microbiological values for pantothenic acid. These included 1:20 liver powder, yeast, wheat bran, and rice bran extract. The apparent pantothenic acid content of these materials as determined by rat assay ranged from 114 to 179% of that found microbiologically (table 2). The addition of vitamin B_{12} to the basal ration did not significantly alter the response of rats to free pantothenic acid nor to 1:20 liver powder (table 3).

[8] R. E. Handschumacher, H. Higgins, T. E. King, G. C. Mueller, L. J. Teply, V. H. Cheldelin and F. M. Strong, unpublished work.

Most of the other products tested yielded essentially the same values by rat assay as by microbiological assay. These included a group of crude materials from yeast or liver, many of which were by-products in a procedure for concentrating coenzyme A.[9] All were high in bound pantothenic acid but contained little or none of the coenzyme (table 2). Two of three preparations of coenzyme A showed equivalent activity by the two types of assay, but the third was more active by rat assay than by microbiological assay, and the discrepancy was similar to that observed with 1:20 liver

TABLE 3

Effect of vitamin B_{12} on a rat growth assay for pantothenic acid

MATERIAL ADDED	3 WEEKS [1]		5 WEEKS [1]	
	No B_{12}	+ B_{12}	No B_{12}	+ B_{12}
	gm	*gm*	*gm*	*gm*
Negative	90	97	104	108
4 μg PA per gram diet	138	134	197	181
8 μg PA per gram diet	170	165	221	223
16 μg PA per gram diet	188	184	240	234
12 mg liver powder (1:20) per gram diet	168	160	224	220

[1] Actual average body weight of each group.

powder (table 2). The sample of the *L. bulgaricus* factor (LBF) tested was as active by rat assay as by microbiological assay.

The analyses for the synthetic phosphates appeared to be of the same order of magnitude for the two types of assay, the rat growth to microbiological ratios averaging 96% for the diphosphate and 92% for the "γ" monophosphate (table 2). Both phosphates showed very little free pantothenic acid activity with *L. arabinosus*. Panthenol was completely inactive for *L. arabinosus*, but in a single experiment it appeared to be as active as free pantothenic acid in stimulating the growth of rats.

[9] See footnote 8, page 365.

DISCUSSION

Earlier workers demonstrated the essential nature of pantothenic acid for rat growth and obtained graded responses at varying dosage levels (Edgar and Macrae, '37; Hitchings and Subbarow, '39; Unna, '40). Likewise, the use of rats has been suggested for pantothenic acid assay (Bacon et al., '43), although the method has not been used as extensively as assays with chicks or microorganisms. One major difficulty has been to provide pantothenate-free supplements carrying other members of the B complex, at least some of which are now available in pure form.

On the present diet the growth of the negative controls usually averaged less than 7 gm per week during the assay period provided the assay was preceded by a preliminary depletion period of two weeks. Essentially similar results were obtained with a commercial vitamin-free casein as with an alcohol-extracted crude casein. The addition of vitamin B_{12} to the basal diet did not alter the growth responses of either the control or the supplemented animals. Results in general were reproducible.

The present results all agree with the conclusion that the pantothenic acid of coenzyme A, of natural fragments of coenzyme A such as the *Lactobacillus bulgaricus* factor, and of possible fragments such as the synthetic phosphates are all at least as available as free pantothenic acid for the growth of rats. The agreement between the two types of assay also extended to concentrates from yeast and liver which were low in coenzyme A but high in other bound forms of pantothenic acid. The inference, therefore, is that these other forms also are fully as active for rats as for *L. arabinosus*.

The only marked disagreements between the results of the rat assay and the microbiological assay were for a group of materials including 1:20 liver powder, whole yeast, wheat bran, one coenzyme A concentrate and possibly rice bran extract, in all of which the rat assay yielded relatively high results. This discrepancy might be explained in several ways.

The extra rat growth may have been due to dietary factors in these materials which were not present in the basal diet; if so, vitamin B_{12} was not the critical factor, since it did not affect the results of the rat assay. Another possibility is that the results of the microbiological assay were too low, possibly because of an incomplete release of the pantothenic acid in these materials into a form that could be used by the assay organism. A third possibility is that the microbiological results were correct and that the high growth response by the rats was due to the presence in these materials of pantothenic acid derivatives particularly active for the animals. This seems unlikely in view of the results with known derivatives (table 2).

SUMMARY

1. Fractions of liver and yeast as well as certain synthetic derivatives of pantothenic acid were assayed microbiologically with *Lactobacillus arabinosus* and biologically by a rat growth method.

2. The results of the rat assays ranged from 114 to 179% of the microbiological values for 1:20 liver powder, yeast, wheat bran and rice bran extracts.

3. Relatively good agreement between the results of microbiological and rat growth assays was obtained for concentrates high in coenzyme A and also for a preparation of the *Lactobacillus bulgaricus* factor.

4. Rats grew approximately as well on the diphosphate and the "γ" monophosphate of pantothenic acid as on the free acid. Panthenol showed no activity with *L. arabinosus*, but in a single assay was as active for rats as pantothenic acid itself.

5. A series of fractions from yeast and liver showed essentially similar activities by rat assay as by microbiological assay. These substances were all high in bound pantothenic acid but low in coenzyme A. The inference, therefore, is that other naturally occurring bound forms of pantothenic acid are completely available to rats.

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VITAMIN B_{12} REQUIREMENT OF MALE WEANLING PIGS [1]

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FOUR FIGURES

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Since the isolation of vitamin B_{12}, extensive research has shown its value in nutrition. Although the amount of experimental work with vitamin B_{12} concentrates and crystalline vitamin B_{12} has been great, relatively little is known about its quantitative requirement by swine. In early work with growing-fattening pigs, Catron and Culbertson ('49) estimated the vitamin B_{12} requirement to be 10 μg per pound of total ration when a standardized vitamin B_{12} concentrate was fed. Lepley ('50), using a commercial concentrate [3] containing 2 mg of vitamin B_{12} per pound of concentrate, obtained 1.67, 1.78 and 1.80 lb. daily gain with growing-fattening pigs fed levels of 0, 5, and 10 μg of vitamin B_{12} per pound of ration. Vohs et al. ('51) concluded that the practical vitamin B_{12} requirement of growing-fattening pigs from weaning to 200 lb. under concrete-drylot conditions does not exceed 5 μg per pound when added to a corn-soybean oil meal ration.

[1] The crystalline vitamin B_{12}, streptomycin sulfate, procaine penicillin G and all vitamins, except vitamins A, D_3 and folic acid, were supplied by Merck and Co., Inc., Rahway, New Jersey. The vitamin D_2 was supplied by Standard Brands, Inc., New York, New York; the aureomycin hydrochloride and folic acid by Lederle Laboratories, Pearl River, New York; and the terramycin hydrochloride by Chas. Pfizer and Co., Inc., Brooklyn, New York.

[2] Journal paper J-1895 of the Iowa Agricultural Experiment Station, Ames. Project 930.

[3] Merck.

He fed a chick-assayed vitamin B_{12} concentrate at different levels and used an additional group of pigs fed crystalline vitamin B_{12} as a reference standard. Neisheim et al. ('50) have shown that the small pig under 8 weeks of age requires approximately 9 μg of vitamin B_{12} per pound of total ration.

The purpose of the present experiment was to determine the vitamin B_{12} requirement of the male pig from weaning to 75 lb., using different levels of crystalline vitamin B_{12} in a corn-soybean oil meal ration.

EXPERIMENTAL PROCEDURE

Six Duroc litters containing 4 male pigs each were alloted at random to the various ration treatments. Three pigs per treatment were started on experiment at 60 days of age. The weight of the pigs in treatments 1 through 4 ranged from 28.5 to 34 lb., and treatments 5 through 8 from 24.5 to 34 lb., or an average of 33.7 ± 0.5 and 29.0 ± 0.3 lb., respectively. During gestation the dams of these pigs received an adequate 16% protein ration containing sources of animal protein, minerals including trace minerals and vitamins A, D_2, riboflavin, niacin and pantothenic acid. For lactation an all-plant ration was fed, fortified only with minerals, including trace minerals and vitamins A and D_2.

After allotment, the pigs were dewormed with sodium fluoride and sprayed with benzene hexachloride. They were then reweighed and started on their respective rations. The pigs were kept in 18-by-40-inch crates with wire floors. Individual self-feeders and watering pans were used. The crates were thoroughly washed three or 4 times daily as an extra precaution in preventing coprophagy. The basal ration, consisting of ground yellow corn and blended soybean oil meal plus a vitamin mixture and minerals, including trace minerals, is shown in table 1.

Levels of 0, 5, 10, and 20 μg of crystalline vitamin B_{12} were added per pound of ration in a soybean oil meal premix. One-half of the pigs on each level of vitamin B_{12} received

10 mg each of aureomycin hydrochloride, streptomycin sulfate, terramycin hydrochloride, and procaine penicillin G.

DISCUSSION AND RESULTS

A summary of the growth results is shown in table 2. There was a slight increase in average daily gain over that obtained with the basal ration as 5, 10, and 20 μg of vitamin B_{12} were added per pound of ration. Only the 20-μg level gave a sig-

TABLE 1

Composition of the basal ration

CHIEF INGREDIENTS		VITAMIN PREMIX NO. 5: AMOUNTS PER POUND OF RATION	
	%		mg
Ground yellow corn	71.0	α-Tocopherol acetate	1.5
		Biotin	0.2
Solvent soybean oil meal		Calcium pantothenate	5.0
(blended)	24.5	Choline chloride	250.0
		Folic acid	0.5
Special steamed bone meal	2.2	Inositol	200.0
		Niacin	10.0
Vitamin premix no. 5	1.0	*p*-Aminobenzoic acid	0.5
		Thiamine	1.5
Iodized calcium carbonate	0.7	Riboflavin	1.0
		Pyridoxine	1.5
Iodized salt	0.5	Pyracin	1.0
		Vitamin K	0.4
Trace mineral mixture[1]	0.1	Vitamin A	300 I.U.
		Vitamin D_2	400 U.S.P. units

[1] Contributed the following to the ration in parts per million: Fe 70, Co 1.6, Cu 4.8, Mn 59, Zn 4.4, and K 7.6.

nificantly ($P = 0.05$) greater gain than the basal ration. The pigs receiving the basal ration including antibiotics but without vitamin B_{12} gained significantly ($P = 0.01$) less than those receiving the basal ration alone. It appears that the antibiotics adversely affected the intestinal flora, including those which synthesize vitamin B_{12} and also those that compete with the pig for vitamin B_{12}. These pigs grew poorly because no vitamin B_{12} was supplied in the ration and apparently vitamin B_{12} synthesis in the intestinal tract was inhibited.

The average daily gain of all the pigs receiving 40 mg of antibiotics per pound of ration was 1.43 lb., which is significantly ($P = 0.01$) greater than the 1.24 lb. gained by those pigs receiving no antibiotics. In this case it is suggested that the antibiotics adversely affected the intestinal flora which compete with the pig for vitamin B_{12}, thus making more of the vitamin B_{12} which was added in the ration available for the pig.

The addition of vitamin B_{12} at all levels with antibiotics produced gains which were greater to a highly significant

TABLE 2

Average daily gains in pounds of individual pigs to 75 lb. live weight

LEVEL OF VITAMIN B_{12} (μg per lb. ration)		0	5	10	20	AVERAGE OF ALL LEVELS
No antibiotics	Litter 30	1.30	1.26	1.29	1.38	
	Litter 40	1.19	1.21	1.23	1.27	
	Litter 120	1.08	1.19	1.23	1.22	
	Average	1.19	1.22	1.25	1.29 [1]	1.24
Antibiotics	Litter 60	1.05	1.52	1.63	1.44	
	Litter 90	1.00	1.56	1.57	1.53	
	Litter 630	0.69	1.55	1.53	1.63	
	Average	0.91	1.54 [2]	1.58 [2]	1.53 [2]	1.43 [2]

[1] Significant at $P = 0.05$.
[2] Significant at $P = 0.01$.

($P = 0.01$) degree than did the basal ration with antibiotics alone. However, there were no differences in the growth response obtained with 5-, 10- and 20-μg levels. Vitamin B_{12} in the presence of antibiotics gave a significantly higher ($P = 0.01$) rate of gain than when fed without antibiotics.

The addition to the ration of vitamin B_{12} alone increased feed efficiency but not significantly. Antibiotics added with vitamin B_{12} gave a significant increase in feed efficiency ($P = 0.01$) of 42 lb. of ration per 100 lb. of gain. Table 3 shows the average daily feed consumption per pig and the feed required per 100 lb. of gain.

A blood sample was obtained from each pig as he reached 75 lb. There were no significant differences in hemoglobin, red and white blood cell counts, or total nitrogen among the treatments. Antibiotics added to the basal ration increased

TABLE 3

Average daily feed consumed per pig and feed required per 100 lb. gain

LEVELS OF VITAMIN B_{12} (μg per lb. ration)	0	5	10	20	AVERAGE OF ALL LEVELS
Average daily feed (lb.)					
No antibiotics	3.20	2.87	3.10	3.00	3.04
Antibiotics	2.10	3.01	3.02	2.94	2.77
Average	2.65	2.94	3.06	2.97	
Feed per 100 lb. gain (lb.)					
No antibiotics	265	236	248	233	246
Antibiotics	235	195	192	193	204 [1]
Average	250	216	220	213	

[1] Significant at P = 0.01.

TABLE 4

Summary of blood data

μG VITAMIN B_{12} PER POUND OF RATION	HEMOGLOBIN, GM PER 100 ML BLOOD	RED BLOOD CELL COUNT (000 OMITTED)	WHITE BLOOD CELL COUNT	TOTAL NITROGEN, GM PER 100 ML	UREA, MG %
No antibiotics					
0	13.6	6,605	20,107	2.83	14.4
5	12.6	4,648	16,983	3.22	15.0
10	14.5	5,560	18,507	3.32	15.6
20	12.9	5,291	19,827	2.75	14.8
Antibiotics (40 mg per pound of ration)					
0	12.7	5,791	13,267	2.99	20.7
5	15.7	7,453	17,427	3.07	9.1
10	12.3	6,953	18,627	3.25	11.1
20	11.9	7,037	15,373	3.07	7.4

blood urea; however, antibiotics plus vitamin B_{12} lowered the blood urea. A summary of these results in given in table 4.

When each pig reached 75 lb. in weight, a 24-hour sample of urine was collected in toluene. The sample was stored in

a freezer at −23°C. Microbiological assay, using *Lactobacillus leichmannii 313* as the assay organism, showed very small amounts of vitamin B_{12} in the urine; however, the trend was upward as the amount of vitamin B_{12} in the feed was increased. Table 5 shows a summary of these results. Feces were also collected at three- to 4-hour intervals and stored in a refrigerator until a 24-hour sample was obtained. The sample was then placed in a freezer at −23°C. until assayed. A summary of the fecal assays is presented in table 6. As in

TABLE 5

Summary of vitamin B_{12} assay of urine

μG VITAMIN B_{12} PER POUND OF RATION	AVERAGE ML URINE IN 24 HOURS	MμG [1] OF B_{12} PER ML	MμG [2] OF B_{12} PER ML	MμG OF B_{12} IN 24 HOURS
No antibiotics				
0	737	1.24	1.50	914
5	600	1.59	1.80	954
10	703	2.76	2.40	1940
20	750	2.79	2.30	2092
Antibiotics (40 mg per pound of ration)				
0	237	0.62	0.83	147
5	657	1.17	1.50	769
10	783	1.78	1.50	1394
20	613	3.43	2.00	2103

[1] Iowa State College, Ames.

[2] Chas. Pfizer and Co., Inc., Brooklyn, New York.

the urine, there was an increase of vitamin B_{12} in the feces as the amount fed increased, except on the 10-μg level with antibiotics. The feeding of antibiotics apparently did not interfere with the assay. This is probably because of the high dilution factor in assaying. Alkali destruction by steaming the samples in 0.2 N potassium hydroxide and paper strip chromatography showed that 97 to 98% of the growth-promoting activity for *Lactobacillus leichmannii 313* was due to vitamin B_{12} and not to thymidine or other alkali-stable growth-promoting factors.

One pig from each treatment was sacrificed to obtain the liver. The vitamin B_{12} assays of the livers are presented in table 7. It will be noted that the vitamin B_{12} storage in the liver increased with the amount of vitamin B_{12} fed.

Pigs receiving the basal ration and antibiotics without additional vitamin B_{12} exhibited vitamin B_{12} deficiency, as evidenced by an extremely rough hair coat, a dermatitis which was not due to mange, and an exudate around the eyes. They were very nervous and irritable. These pigs also had a ten-

TABLE 6

Summary of vitamin B_{12} assay of feces

μG VITAMIN B_{12} PER POUND OF RATION	AVERAGE GM OF FECES IN 24 HOURS	μG [1] OF B_{12} PER GRAM	μG [2] OF B_{12} PER GRAM	μG OF B_{12} IN 24 HOURS
No antibiotics				
0	229	0.85	0.86	195
5	206	0.95	0.99	196
10	486	1.00	1.10	486
20	411	1.35	1.26	555
Antibiotics (40 mg per pound of ration)				
0	130	0.80	0.80	104
5	388	1.15	1.24	446
10	492	0.70	0.52	344
20	263	2.50	2.07	658

[1] Iowa State College, Ames.

[2] Chas. Pfizer and Co., Inc., Brooklyn, New York.

dency to be constipated. Pictures of a set of litter mates fed 0, 5, 10, and 20 μg of vitamin B_{12} plus 40 mg of antibiotics per pound of ration are shown in figures 1 through 4. These pictures were taken 24 days after the pigs were started on the experiment.

One pig which showed vitamin B_{12} deficiency and exhibited growth stasis was injected intraperitoneally with 10 μg of vitamin B_{12} when he weighed 75 lb. He was kept on the same ration for one week and then 10 μg of vitamin B_{12} were added to each pound of ration for three more weeks. During the

4-week period the pig gained 35 lb. This was an average daily gain of 1.25 lb. as compared with the previous average daily gain of 0.69 lb. before he received vitamin B_{12}.

SUMMARY AND CONCLUSIONS

Six sets of 4 male litter-mate pigs, three per treatment, were individually fed and watered ad libitum from weaning to a weight of 75 lb. in wire floored crates. The pigs were fed a fortified corn-soybean oil meal ration to which were added

TABLE 7

Summary of vitamin B_{12} assay of liver

μG VITAMIN B_{12} PER POUND OF RATION	TOTAL GM	μG [1] OF B_{12} PER GRAM	TOTAL μG OF B_{12}
No antibiotics			
0	794	0.057	45
5	681	0.082	56
10	735	0.152	112
20	796	0.130	103
Antibiotics (40 mg per pound of ration)			
0	732	0.056	41
5	687	0.083	57
10	686	0.144	99
20	667	0.133	89

[1] Chas. Pfizer and Co., Inc., Brooklyn, New York.

0, 5, 10, and 20 μg of crystalline vitamin B_{12} per pound of ration. The ration treatments were subdivided, with one-half of the pigs receiving 40 mg of antibiotics per pound of ration.

Vitamin B_{12} alone produced only slight increases in daily weight gain but in the presence of antibiotics the increases were highly significant. There were no significant differences in responses among the added levels of vitamin B_{12} as judged by the weight gain of the pigs. The addition of an antibiotic mixture to the ration increased average daily gains and feed efficiency significantly. There were no differences among

treatments with respect to hemoglobin, red and white blood cell counts or total nitrogen in the blood. Antibiotics alone increased blood urea; however, when vitamin B_{12} was added the blood urea was lowered. There was an increase of vitamin B_{12} in the urine as the amount in the ration increased. This was also true with the feces, except for the 10-μg level with added antibiotics. Similarly, there was an increase in the vitamin B_{12} content of the liver as the levels of vitamin B_{12} in the rations were increased.

The weanling pig needs vitamin B_{12} for optimum growth and feed efficiency. With intestinal flora control through the use of a combination of antibiotics, the vitamin B_{12} requirement of the weanling pig is 5 μg or less per pound of ration when the antibiotics are added to a corn-soybean meal diet.

It is suggested that a mixture of antibiotics similar to the one used here might be used in studying the minimum requirement of other vitamins which may be synthesized in the intestinal tract of the pig.

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PLATE 1

EXPLANATION OF FIGURES

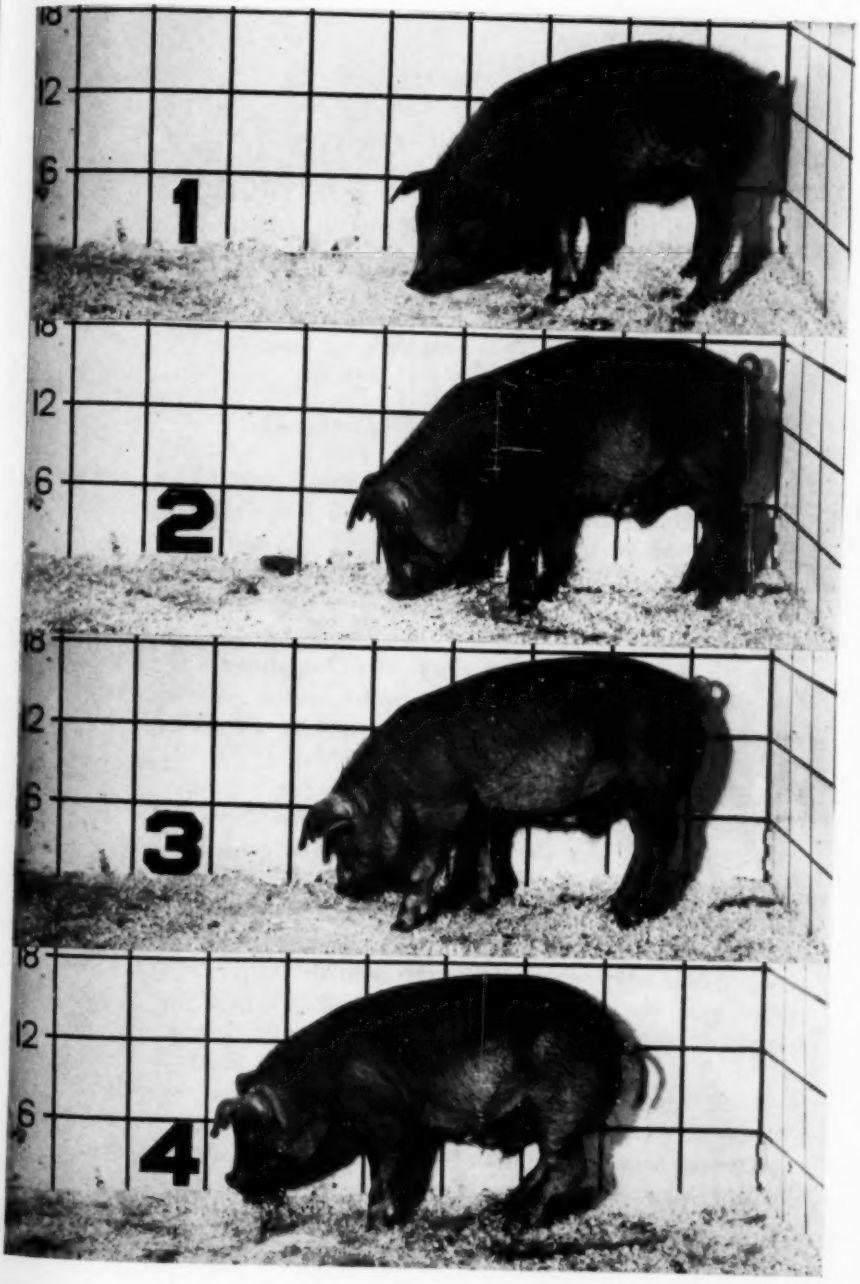
This set of litter mates shows the typical response obtained with different levels of crystalline vitamin B_{12}.

1 Pig 64 received the basal ration plus 40 mg of antibiotics per pound of ration. During the 24 days from the start of the experiment until these pictures were taken, this pig gained 21 lb. Note the extremely rough hair coat, dermatitis and tucked up flank. He became very nervous and irritable.

2 Pig 62 received the basal ration plus 40 mg of antibiotics and 5 μg of vitamin B_{12} per pound of ration. He gained 37 lb. during the first 24 days on experiment.

3 Pig 61 received the basal ration plus 40 mg of antibiotics and 10 μg of vitamin B_{12} per pound of ration. He gained 34 lb. during the first 24 days on experiment.

4 Pig 60 received the basal ration plus 40 mg of antibiotics and 20 μg of vitamin B_{12} per pound of ration. He gained 35.5 lb. during the first 24 days on the experiment. All pigs except number 64 had normal hair coats and skin. They did not show any signs of nervousness or irritability.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THE ABSORPTION OF IRON FROM SPINACH BY SIX YOUNG WOMEN, AND THE EFFECT OF BEEF UPON THE ABSORPTION

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In recent years spinach has fallen into disrepute, at least in the popular mind, as a source of iron in the diet. The scientific basis for this attitude may be found in the fact that when the α,α′-bipyridine test for so-called "available" iron in food was developed, spinach, one of the foods highest in total iron content, was found to be one of the lowest in percentage of "available" iron. Only 20% of its iron was found to be available, as judged by either the α,α′-bipyridine test or by hemoglobin regeneration in rats made anemic on a milk diet (Sherman, Elvehjem and Hart, '34). The results of 6 studies reported since that time on the feeding of spinach to animals are varied. Two of the most recent of these reports are those of Ruegamer et al. ('46) and Pye and MacLeod ('46). In the former study, dogs made anemic by bleeding were shown to utilize 10 to 20% of the iron of dried spinach for hemoglobin formation, as compared with 50% utilization from ferric pyrophosphate. In the latter study the absorption of iron, as measured by the increase in total body iron content in normal rats, was 26% from raw spinach, 31% from cooked spinach, and 42% from $FeCl_3$.

Whether the performance of human beings can be accurately predicted from animal work with spinach is questionable. Iron in food, except for that which is in hematin, is present primarily in the ferric state. Moore ('47) has

demonstrated that different species may vary in the degree to which they utilize the ferric form, and man absorbs 4 to 5 times as much of a dose of ferrous as of ferric salts.

The studies which have been made on the absorption of iron from spinach by human subjects, so far as we have been able to ascertain, have been limited to infants. Infants fed pureed spinach were studied by Schlutz, Morse and Oldham ('33a, b, '37) and by Stearns and Stinger ('37). Neither group was able to demonstrate absorption of iron from the spinach. The response of infants, however, may not be typical of adults, whose digestive tracts are more accustomed to foods high in roughage. The problem of the absorption of iron from spinach by the human adult is of practical importance because of the relatively high concentration of the mineral in it and the wide availability of the vegetable. For this reason, the present study was planned to determine the absorption of iron from spinach by normal young women.

The plan of the study was to determine the amount of fecal iron resulting from a basal diet containing just enough iron to cover the body's needs, then to add a serving of spinach per day to the diet and again determine the amount of fecal iron. Several workers (McCance and Widdowson, '38; Hahn et al., '39; Little, Power and Wakefield, '45) have demonstrated that there is little or no excretion of iron through the intestinal wall into the gastrointestinal tract. The iron in the feces is that which has not been absorbed from the food as it passes through the tract, except for a small amount which may originate from bile which was not reabsorbed or from cells sloughed from the intestinal wall. Therefore, the difference in the amount of iron appearing in the feces before and after the addition of spinach to the diet would represent the amount of iron from the spinach which had not been absorbed. Since the amount of iron contained in the spinach was known, the amount absorbed could be determined.

The assumption was made that if any iron were derived from intestinal sources previously mentioned, it would be constant in amount, would occur in the feces from both

diets, and would consequently introduce no error into the calculated difference in absorption.

The present study was also planned to test a theory suggested as a result of previous work in this laboratory. Johnston, Frenchman and Boroughs ('48) studied the absorption of iron from beef in the manner outlined above and found that when additional beef was incorporated in the diet, the iron absorption was increased to the extent of one-third to one-half the amount of iron in the beef. These authors postulated that the additional iron absorbed may have represented not only iron from the beef but improved absorption from the entire food mixture due to the presence of beef. In an attempt to determine, in the present study, whether beef could improve the absorption of iron from the food mixture, the spinach was fed for one period in the evening meal, which contained a serving of beef, and for another period in the breakfast, which contained no animal protein of any kind. If the beef improved the absorption of iron from the entire food mixture, a higher absorption should result during the period when the spinach was served with beef than during the period when it was not.

PROCEDURE

The subjects were 6 college women 20 to 31 years of age. Each was judged suitable for the experiment on the basis of a physical examination, blood analysis, previous dietary history, and ability to cooperate intelligently.

The study was divided into three periods of 4 weeks each. For the week preceding the beginning of the study the subjects selected their diets to approximate closely the nutritive value of the basal diet to be fed, so that this week might serve as an adjustment period. The basal diet was served for 4 weeks; for the following 4 weeks 120 gm of spinach were added to the evening meal of three subjects and to the breakfast of the remaining three; for the last 4-week period the time at which the spinach was served was reversed for the subjects.

The basal diet was composed of a wide variety of common foods and was planned to meet the National Research Council recommended daily allowances for all nutrients except calcium and iron. Calcium was fed at a level of approximately 0.8 gm per day and iron at a level of approximately 7 mg per day. This amount of iron was calculated to be sufficient to cover the body's needs and yet provide a critical level at which differences in absorption would be demonstrable. Approximately half the thiamine was supplied as thiamine hydrochloride. The menus were planned for one week and repeated during each of the 12 weeks. Each evening meal contained a serving of beef equivalent to 110 gm raw, lean meat; otherwise, the morning and evening meals were quantitively and qualitatively identical. The other foods served were fruit, a cereal product without milk, and coffee; all animal protein was avoided to preclude the possibility that it might exert the same effect as beef. Such items as milk, cheese, and eggs were served at the noon meal, as were the vegetables. Distilled water was used for drinking and in food preparation. Unenriched flour was used to make all bread and pastries; these were made with water. Foods allowed ad libitum were butter, *c.p.* sugar, *c.p.* salt, pepper, and bread at the noon meal; a limited amount of coffee or a specified cola drink was permitted after 10 P.M.

In order that the food should be as constant in composition as possible throughout the study, all fruits and vegetables except citrus fruits were bought in one lot and were either frozen or stored. Eggs were mixed and frozen in weighed portions. All the beef was frozen raw; the ground meat was from one lot, while the steaks served during each period were from comparable locations on the same animals, and the roasts served each week of the last two periods were from opposite sides of the same animals.

The spinach was a commercial frozen product which assayed high in iron. Each lot was partially defrosted, cooked quickly in a saucepan, drained 10 minutes in an aluminum strainer, and weighed on a trip balance into half-cup freezer cartons.

The cartons were frozen; one was taken for analysis, and the remainder were divided into two batches to be used on corresponding days in the two periods. For serving, the frozen spinach was defrosted in a custard cup and heated in the oven. The iron content varied somewhat from package to package, and the lots were selected to yield a similar mean iron intake for each week.

Great care was taken to prevent contamination of the food with iron, and to assure that the composition of the food for each subject and the analyzed samples were identical. The food was prepared in pyrex and aluminum utensils; the kitchen was kept free from sources of rust. In the preparation of mixed dishes, the ingredients were weighed into individual casseroles before being cooked. Dietetic scales were used for weighing.

Duplicate servings of each food were taken each day, mixed in mechanical blenders and aliquot portions taken for duplicate weekly composites. The spinach, milk, and foods allowed ad libitum were analyzed separately. The feces, which were marked by carmine given at the first breakfast of each week, were made into weekly composites. All composites were heated with HCl and stored in glass containers.

The food and feces were wet-ashed with nitric and sulphuric acid, and the nitrosyl sulphuric acid hydrolyzed by boiling with water according to the method of Roberts, Beardsley and Taylor ('40). The thiocyanate method of Stugart ('31) was used for the determination of iron, and the colors were read in an Evelyn Photoelectric Colorimeter.

Samples of blood were taken by venipuncture before the study began and during the first week of the second and third periods. Hemoglobin determinations were made in duplicate by the method of Evelyn ('36) and red blood cell counts were made in duplicate on blood from each of two pipettes.

RESULTS AND DISCUSSION

The range of hemoglobin values for the subjects throughout the study was 12.8 to 14.9 gm per 100 ml of blood, and

TABLE 1

The intake, fecal excretion and absorption[1] of iron per week for 6 subjects on three diets

DIET	SUBJECT	WEEK 1			WEEK 2			WEEK 3			WEEK 4		
		Intake	Excretion	Absorption	Intake	Excretion	Absorption	Intake	Excretion	Absorption	Intake	Excretion	Absorption
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Basal	D.C.	44.21	41.30	2.91	45.42	38.68	6.74	45.79	39.58	6.21	43.91	40.20	3.71
	L.O.	44.74	36.84	7.90	45.97	32.23	13.74	46.33	40.26	6.07	44.59	42.97	1.62
	B.M.	44.58	44.20	0.38	45.55	42.57	2.98	46.31	37.51	8.80	43.88	39.87	4.01
	J.S.	44.37	50.64	— 6.27	45.55	38.04	7.51	45.82	30.04	15.78	43.81	41.17	2.64
	D.D.	45.26	36.70	8.56	46.23	54.32	— 8.09	46.67	69.37	— 22.70	44.83	36.83	8.00
	J.M.	43.90	41.31	2.59	44.88	33.52	11.36	45.31	42.65	2.66	43.41	43.57	— 0.16
Basal	D.C.[2]	81.86	74.10	7.76	78.54	68.24	10.30	80.32	73.82	6.50	80.70	71.04	9.66
plus	L.O.[2]	82.20	78.32	3.88	79.15	73.28	5.87	80.65	68.48	12.17	80.94	86.23	— 5.29
spinach	B.M.[3]	80.56	77.98	2.58	80.55	62.44	18.11	82.26	73.52	8.74	80.05	69.55	10.50
apart	J.S.[3]	80.03	83.36	— 3.33	80.04	71.36	8.68	81.62	52.76	28.86	79.51	69.96	9.55
from	D.D.[3]	82.75	61.75	21.00	79.42	88.50	— 9.08	81.17	55.11	26.06	81.56	70.44	11.12
beef	J.M.[3]	79.68	63.97	15.71	79.50	75.49	4.01	81.24	77.72	3.52	79.04	64.31	14.73
Basal	D.C.	80.13	68.22	11.91	80.04	66.62	13.42	81.91	76.91	5.00	79.59	74.08	5.51
plus	L.O.	80.81	65.32	15.49	80.49	69.35	11.14	82.31	66.87	15.44	80.06	79.58	0.48
spinach	B.M.	82.45	61.23	21.22	79.10	74.92	4.18	80.55	63.71	16.84	81.15	68.47	12.68
together	J.S.	81.72	61.06	20.66	78.45	76.62	1.83	80.27	56.82	23.45	80.56	77.23	3.33
with	D.D.	81.08	63.91	17.17	80.92	58.20	22.72	82.64	62.74	19.90	80.39	75.13	5.26
beef	J.M.	81.32	102.70	— 21.38	78.01	91.13	— 13.12	79.84	100.18	— 20.34	80.16	88.25	— 8.09

[1] Calculated as food iron minus fecal iron.
[2] Subjects eating spinach first together with beef.
[3] Subjects eating spinach first apart from beef.

the red cell counts ranged from 4.22 to 5.02 M per mm^3. These were normal blood values for young women and indicated that normal iron absorption by these subjects could be anticipated.

TABLE 2

The mean absorption of iron per day for 6 subjects for 28 days on each of three diets

SUBJECT	ABSORPTION OF IRON [1]				
	Basal diet	Basal diet plus spinach fed apart from beef		Basal diet plus spinach fed together with beef	
	6.44 mg mean total intake	11.50 mg mean total intake	5.04 mg intake from spinach	11.51 mg mean total intake	5.04 mg intake from spinach
	mg	*mg*	*mg*	*mg*	*mg*
D.C.	0.70	1.22	0.52	1.28	0.58
L.O.	1.05	0.57	— 0.48	1.52	0.47
B.M.	0.58	1.43	0.85	1.96	1.38
J.S.	0.71	1.56	0.85	1.76	1.05
D.D.	— 0.51 [2]	1.75 [2]		2.32 [2]	
J.M.	0.59 [2]	1.36 [2]	0.77 [2]	— 2.24 [2]	
Mean	0.76	1.20	0.43	1.63	0.87
Standard error [3]	0.101	0.220	0.317	0.147	0.211

[1] The figures for total absorption from each diet were obtained by subtracting fecal iron from food iron, and are somewhat low if iron from intestinal sources was present in the feces. The iron absorbed from spinach was calculated as the difference between absorption on the basal diet and on the basal diet plus spinach; it is not subject to the above error.

[2] Omitted from the mean.

[3] The standard error of the mean as calculated by the formula

$$s_{\bar{x}} = \sqrt{\frac{s^2}{n}},$$

in which s^2 = variance and n = number of observations.

The difference between the intake and fecal excretion of iron each week for each subject was calculated (table 1). The mean difference per day for the 28 days on each diet was calculated for each subject (table 2).

For subject D.D. during the basal period and for subject J.M. for the period on spinach eaten at the same meal with beef, the mean daily fecal excretion was greater than the in-

take. If the feces contain only the residue from the food except for the small amount of iron from intestinal sources, the amount of iron in the feces should not exceed that in the intake. The origin of the excess iron in the feces is problematic; the authors do not feel that the assumption that the excess iron had been excreted from the tissues is valid in the light of previously cited work, which has failed to show excretion of iron into the intestine. To find more iron in the feces than was in the diet is possible for a collection period of one week when there has been a spread of the fecal marker in the intestine and the feces which do not belong to that week are included in the composite. There are several possible causes of excess iron in the feces over an extended period of time: (1) contamination of the food or of the feces with iron may occur. Sources of iron contamination can be controlled during chemical analyses; they are difficult to eliminate in food preparation and from the subject's environment; (2) bleeding into any portion of the gastrointestinal tract will provide an unknown source of iron; (3) iron is added to the digestion mixture by bile. In a study of one normal man Rechenberger and Pollack ('44) found that about 1 to 3 mg of iron a day entered the gastrointestinal tract by way of the bile, and that it was completely reabsorbed. Whether under some conditions significant quantities of bile-iron are not reabsorbed remains a problem for investigation. Any one, or a combination, of the conditions listed above, as well as unknown factors, may have contributed to the results obtained for subjects D.D. and J.M. For subject D.D. contamination seems a possible cause of the large amount of fecal iron in week 3 of the basal period; in the case of subject J.M., contamination for 4 consecutive weeks for one individual seems improbable. An examination for blood in the fresh feces of subject J.M. was made a month after the close of the study; none was found. The only known deviation from normal in the history of subject J.M. was that her menstrual period just prior to the beginning of the study was the first in several months; her sense of well-

being was not impaired during this time, and during the study her menstrual cycles were regular. Because, in the authors' opinion, the data for subjects D.D. and J.M. cannot be accepted without great reservation as typical responses to the conditions of the study, emphasis in the following discussion of the results is placed primarily upon the data obtained for the remaining 4 subjects.

TABLE 3

Analysis of variance for absorption[1] of iron by 4 subjects on three diets

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F	F_{05}	F_{01}
Weeks	3	135.342			
Diets	2	149.660	6.35	3.55	6.01
Subjects	3	14.475			
Weeks × diets	6	76.677	3.25	2.66	4.01
Weeks × subjects	9	72.866	3.09	2.46	3.60
Diets × subjects	6	23.330	0.99	2.66	4.01
Diets × weeks × subjects	18	23.570			
Total	47				
ANALYSIS OF ABOVE MEAN SQUARE ATTRIBUTABLE TO "DIETS"					
Basal versus the 2 diets containing spinach	1	227.175	9.64	4.41	8.28
Diet containing spinach fed with beef versus diet containing spinach fed apart from beef	1	72.144	3.06	4.41	8.28

[1] Absorption in this case is food iron minus fecal iron.

The mean iron content of the basal diet of the 4 subjects during the first period of 28 days was 6.44 ± 0.019 mg per day. The mean for the total intake of iron for the period when the spinach was fed apart from beef was 11.50 ± 0.018 mg daily, and for the 28 days when spinach was fed together with beef, 11.51 ± 0.023 mg. Of this total, the mean daily amount contributed by the spinach was 5.04 mg. The daily ingestion of a serving of spinach did not cause discomfort for any subject; there was no diarrhea nor any visible change

in the consistency of the stools. Attitudes toward eating the spinach at either time of day ranged from indifference to enjoyment.

An analysis of variance was made on the weekly differences between the iron intake and fecal iron to determine the significance of the variations in absorption which were obtained for the 4 subjects for the 4 weeks on three diets (table 3). The variation attributable to the different diets was found to be significant at the 1% level. Further analysis of this factor indicated that the significant difference lay between the basal diet and the basal diet with spinach added. The difference was significant at the 1% level, indicating that sufficient iron had been absorbed from the added spinach to increase significantly the total amount of iron absorbed. When data for the 6 subjects were included in the analysis of variance, the F value for the three diets indicated no significant difference among diets. However, a comparison of the basal diet with the basal diet plus spinach showed a difference in absorption significant at the 5% level, the F value obtained being 5.37, while values for F necessary for significance at the 1 and 5% levels were 4.17 and 7.56, respectively.

The analysis of variance indicated that there was no significant difference between the total absorption for the 4 subjects from the diet when spinach was eaten apart from beef and when spinach was eaten together with beef (table 3). The amount of iron absorbed from the spinach in each of the two diet periods was calculated by subtracting the difference between food and fecal iron in the basal period from the difference in food and fecal iron in each period when spinach was fed (table 2). Subjects D.C., L.O., and D.D. had spinach first for dinner, and subjects B.M., J.S., and J.M. had spinach first for breakfast. This was done in order that both periods when spinach was fed would be affected equally by any change in absorption due to an increase in

the amount of iron in the diet, or by any effect the ingestion of spinach over a prolonged period might have. Inspection of weekly values for the two groups of subjects failed to show any variations attributable to this difference. For the two diet periods subject D.C. absorbed approximately the same amount, and subjects L.O., B.M., and J.S. absorbed a larger amount when the spinach was fed together with the beef. Subject D.D., whose values are not included in the statistical analysis, also absorbed more iron when the spinach was fed with the beef. For the 4 subjects the mean amount of iron absorbed from the spinach fed apart from the beef was 0.43 ± 0.317 mg, and from the spinach fed with the beef was 0.87 ± 0.211 mg per day.

The results of this study do not demonstrate that an improvement in the absorption of iron from the diet can be brought about by the presence of beef in the food mixture. There remains the possibility that the method employed in this study was not sufficiently sensitive to detect existing differences. The large variations obtained in excretion from week to week would make necessary either a longer period of observation or more subjects to demonstrate small differences in absorption. Any effect beef may have exerted as a reducing agent for the iron was minimized by the inclusion of citrus or other fruit in every meal in which spinach was fed, since citrus fruit is an efficient reducing medium for iron in the stomach (Bergeim and Kirch, '49). Also, if it so happened that the spinach fed in the morning came in contact with the products of digestion of beef in the gastrointestinal tract, the effect of the beef would have been masked.

The mean daily absorption of iron from spinach by the 4 subjects was calculated for the total of 8 weeks when spinach was fed, since there was no statistically significant difference in the amount of iron absorbed during the two 4-week periods. The mean amount of iron absorbed per day for the 8 weeks was 0.66 ± 0.248 mg. This represents 13% of the 5.04 mg of iron contained in a serving of spinach weighing 120 gm. The importance of an absorption of 0.66 mg is ap-

preciated when this amount is compared with the estimated required retention for young women of 1.2 mg per day (Frenchman and Johnston, '49).

An estimate of the total amount of iron absorbed from the basal diet and from the basal diet plus spinach has been obtained in each case from the difference between the amount of iron in the feces and the amount in the food (table 2). This method of calculation fails to consider the possible presence in the feces of a small amount of iron from intestinal sources, and if such iron was present the following figures for absorption are somewhat low. The mean for the amount of iron absorbed by the 4 subjects from the basal diet was 0.76 ± 0.101 mg per day, while the mean for the amount of iron absorbed for the 8 weeks when spinach was fed with the basal diet was 1.41 ± 0.159 mg per day. Expressed as percentage of the intake, the amount of iron absorbed from the basal diet and from the basal diet with spinach added was 12% in each case. If the values for subjects D.D. and J.M. are included, the mean amount of iron absorbed from the basal diet was 0.52 ± 0.217 and from the basal diet plus spinach 1.21 ± 0.360 mg.

The comparative value of spinach as a source of iron for the human adult may now be estimated. The only single food for which comparable data have been obtained is beef. The beef used by Johnston, Frenchman and Boroughs ('48) in their study contained approximately half as much iron per gram of lean meat as did the spinach used in the present study. A mean absorption of 41% of the 3.4 mg of iron from beef was obtained, as contrasted to a mean absorption of 13% from the 5.04 mg of iron from spinach. As compared with meat, either the digestibility of the spinach or the form in which the iron occurs in it is not as conducive to absorption; the difference does not result from the oxalate content of spinach, since ferrous and ferric oxalate, unlike calcium oxalate, are soluble salts. The larger amount of iron per gram in spinach reduces but does not eliminate the superiority of beef as a source of iron.

Comparison of the absorption of iron from spinach with that from the diet as a whole is more favorable. A number of figures are reported in the literature for the mean absorption of iron from a mixed diet, each calculated in the same manner as that described above (food minus feces) and subject to the same correction. For the study of beef which has been discussed (Johnston, Frenchman and Boroughs, '48) absorptions of 11% and 21% were obtained before and after additional beef was incorporated into the diet; Schlaphoff and Johnston ('49) reported a mean absorption by adolescent girls of 14% and 13% for diets containing 7 and 11 mg of iron, respectively; Widdowson and McCance ('42) found a mean absorption of 12.5%. To these data may be added the finding of the present study of a mean absorption of 12% of the total iron in the diet. Since the mean absorption of the iron contained in spinach was calculated to be 13%, the conclusion is drawn that the iron of spinach is as well absorbed as the iron present in a diet comprised of a wide variety of foods. In actual content of iron, spinach exceeds the majority of other foods. These two considerations should lead to the acceptance of spinach as a valuable source of iron in the diet of the adult person.

The analysis of variance (table 3) yielded unexpected significant interactions between weeks and diets and between weeks and subjects. Possible causes for these were sought. The interaction between weeks and subjects was influenced particularly by subjects L.O. and J.S. Subject L.O. consistently absorbed least well in the 4th week of each diet, a time which closely approximated her menstrual period. Subject J.S. on two diets absorbed least during the first week, which was her menstrual period, and always absorbed exceptionally well each third week. Subject D.C. was fairly consistent from week to week; her rate of absorption reached a peak the second week of each diet, which was also the second week after menstruation. Analysis of the data obtained for variations in absorption due to the menstrual cycle is complicated by the fact that fecal collection periods cor-

responded only roughly to menstrual periods; also, the error in a single stool separation, upon which such comparisons depend, can be large. Only a suggestion of a possible connection can be made. The wide fluctuations in excretion from week to week on a constant intake (table 1), so characteristic of iron and not of calcium, which behaves similarly in many ways, may possibly prove to be the expression of a variable physiological state under hormonal control.

The significant interaction between weeks and diets resulted primarily from a higher mean absorption during the first week when spinach was eaten in the presence of beef. The difference was contributed largely by the subjects for whom it was the 5th week of spinach feeding, subjects B.M. and J.S., whose iron excretions were unusually low the first week and unusually high the second week when spinach was fed with beef. Poor fecal separation between the first and second week could account for this difference.

SUMMARY

Data are presented for the intake and fecal excretion of iron by 6 young women on three controlled diets. More iron was found in the feces of two subjects for one 4-week period than was in the diet; reasons are given for not including these data in evaluating the performance of the group.

The mean amount of iron absorbed daily by the remaining 4 young women over a period of 8 weeks from 120 gm of cooked spinach containing 5.04 mg of iron was found to be 0.66 ± 0.248 mg, or 13% of the amount present. Since this percentage compares favorably with the percentage of iron absorbed from diets composed of a wide variety of foods, and since the iron content of spinach is higher than that of a majority of foods, spinach may be regarded as a valuable source of iron for young women.

The mean absorption of iron was greater in the period when the spinach was fed in the presence of beef for 4 of the 5 subjects on whom data were obtained. The difference in the amount of iron absorbed from the spinach when fed

away from or in the presence of beef was not statistically significant, however, and on the basis of the data obtained in this study beef cannot be said to increase the amount of iron absorbed from spinach.

The total amount of iron absorbed from the 6.44 mg of iron in the basal diet and from the 11.51 mg in the basal diet with spinach added was estimated to be 12% in each case. This estimate is somewhat low if the feces contain a measurable quantity of iron from unreabsorbed bile and sloughed-off cells from the intestine.

A possible relationship between the menstrual cycle and iron absorption is suggested by the limited data obtained.

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THE SUPPLEMENTAL VALUE OF CYSTINE AND METHIONINE FOR LOW PROTEIN (CASEIN) DIETS FED THE YOUNG WHITE RAT [1]

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The nutritive value of the proteins of milk, particularly casein and casein supplemented by amino acids, has been a subject of special interest since the pioneer work of Osborne and Mendel ('15). Their results demonstrated that good growth of the young white rat was obtained with higher levels (15 to 18%) of casein, and that with lower levels (9%) growth was not satisfactory but could be significantly improved by the addition of small amounts of cystine. At the time of these and later experiments (see Lewis, '40–'41), cystine, which is very low in casein (0.3 to 0.4%), was the only amino acid containing sulfur of proved occurrence in the protein molecule. Subsequent to the discovery of a second sulfur-containing amino acid in proteins, methionine, this amino acid was also shown to be effective in promoting the growth of young white rats fed diets low in their content of casein (and cystine; Jackson and Block, '32). A metabolic relationship between these two amino acids was suggested and later demonstrated. Rose and his co-workers (Rose, '37; Womack, Kemmerer and Rose, '37; Womack and Rose, '41), who used diets containing mixtures of amino acids rather than protein, reported that methionine was indispensable and cystine dis-

[1] These studies were supported in large part by grants from the National Dairy Council.

pensable, and demonstrated further that these two amino acids were not interchangeable in metabolism.

The accuracy of the early observations and of many of the subsequent studies was limited by lack of uniformity of the protein preparations, the variety of the sources of vitamins, and the questionable purity of other dietary components, all of which make the interpretation and exact repetition of these earlier studies difficult. In addition, variations in the technic of individual experiments and in analyses of the results are such that comparisons of different series of data are not satisfactory.

The present experiments were designed to eliminate variables other than dietary protein (chiefly casein) or amino acids which would influence the growth of young white rats, and to provide for such additions of amino acids as might be important for a diet low in its content of casein. It was of interest to determine whether, after the basal requirements for methionine were met, it would be economical from the standpoint of nutrition (growth) for the remainder of the sulfur-containing amino acid requirement to be supplied by cystine. By what distribution of cystine and methionine in the diet would most effective utilization of the sulfur-containing amino acids for growth be achieved? Since we were interested primarily in the amino acids with reference to their sulfur content, and in the relative efficiency of the cystine present in the diet and the cystine synthesized from the sulfur of methionine, we have in all cases included choline chloride in our diets. Thus approximately 10% of the methyl groups of our basal diet (7.5% casein) were present in the methionine of the protein. It was hoped that this diet would minimize the need for methyl groups derived from methionine and make it possible to evaluate methionine requirements for growth.

EXPERIMENTAL

Young male white rats of the Wistar strain in litter units and weighing from 50 to 60 gm were used. They were fed the

various diets ad libitum for 28 days, and food intakes and weight changes were recorded.

The basal diet (casein) had the following percentage composition: casein,[2] 7.5; sucrose, 41.4; glucose monohydrate, 21.1; cellulose,[3] 5; salt mixture (modified Osborne-Mendel), 4; vitamin mixture (in sucrose), 1; corn oil, 17; and cod liver oil, 3. Choline chloride (0.273 gm) was added to each 100 gm of the diet and 100 mg of dried liver extract [4] were fed to each rat daily. The amino acid supplements replaced an equivalent amount of carbohydrate.

The vitamin mixture contained: thiamine hydrochloride, 37 mg; pyridoxine hydrochloride, 37 mg; riboflavin, 75 mg; calcium pantothenate, 300 mg; *p*-aminobenzoic acid, 300 mg; and nicotinic acid, 375 mg. These were mixed with sufficient sucrose to give 100 gm of the vitamin mixture, which was incorporated into the basal diet as indicated. L-Cystine and DL-methionine showed satisfactory purity as established by analyses for nitrogen and sulfur.

In calculating the amino acid content of our diets, the values of Brand ('46) and Williamson ('44) for casein and lactalbumin were used. Since our preparations were fed as purchased and the analyses cited are calculated on an ash-free, moisture-free basis, we have calculated the amino acid values on the basis of the nitrogen content of our proteins (casein, 14.1%, and lactalbumin, 11.7%). The D- and L-isomers of methionine were considered to be equally available to the rat for purposes of growth (Jackson and Block, '38).

The basal level of casein (7.5%) was chosen as one which would permit slow growth in young rats. When commercial lactalbumin [5] replaced casein in the basal diet,[6] 9 gm of lactal-

[2] Labco vitamin-free. We wish to express our sincere appreciation to Mr. W. A. Wentworth of the Borden Company for generous gifts of the casein and lactalbumin used in these experiments.

[3] Ruffex.

[4] Lilly.

[5] See footnote 2.

[6] This low protein diet containing 7.5% of casein will hereafter be referred to in the discussion as the *basal diet*.

bumin, equivalent in nitrogen content to 7.5 gm of casein, were added, and 1.5 gm of sucrose were removed from the diet. Sucrose (cane sugar) and glucose (Cerelose) were used as sources of carbohydrate in view of evidence (Mannering, Orsini and Elvehjem, '44) that the more highly refined sugars are less conducive to the synthesis of vitamins and other growth factors in the intestinal tract than starch or dextrin. It was thus hoped to keep a potential variable factor, synthesis by intestinal microorganisms, at a minimum. When lactalbumin replaced casein as the protein of the basal diet, the amount of choline chloride added to the diet was so adjusted that the number of methyl groups (109 mg) was essentially the same in both diets.

In the experiments with casein, the sulfur-containing amino acid supplements were added in such amounts that the organic sulfur content of the diets [7] was 185 mg% (except in groups VIII and IX). The character of the amino acid supplements is shown in table 1. When both cystine and methionine were added as supplements to the basal diet, the total organic sulfur still remained at the level of 185 mg%, but the distributions of extra sulfur between supplementary cystine and methionine were in ratios of 1:1 (group IV), 2:1 (group V) and 1:2 (group VI), respectively. Since the results obtained by varying the proportions of supplementary cystine and methionine between these limits did not differ materially, the values for these three groups (IV to VI inclusive) have been combined into a single group (VII) in table 1. In the diet of group VIII the cystine content was increased approximately 20-fold, while the amount of methionine was doubled, and in that of group IX the cystine content was increased 10-fold and the methionine 4-fold. Thus not only the total organic sulfur of the diet but also the distribution of the sulfur varied in these two groups. When the two amino acids were added to the lactalbumin-containing diet (group X), the sup-

[7] Since the content of vitamin mixture and liver extract in the diet was the same in each experiment, we have not included the sulfur present in these in calculating the organic sulfur of the diet.

TABLE 1

Influence of supplementary methionine and cystine added to low protein diets (7.5% casein or the equivalent in lactalbumin) on growth and protein efficiency in young white rats over a 28-day period

GROUP	CASEIN [1]									LACTALBUMIN [1]		
	I Basal	II	III	IV	V	VI	VII [2]	VIII	IX	X	XI	XII
Number of animals	53	24	19	14	14	14	42	12	11	38	17	3
Cystine supplement [3]		500		250	333	167		500	250		500	
Total cystine	27	527	27	277	360	194		527	277	218	718	218
Methionine supplement			621	310	207	414		207	621			621
Total methionine	209	209	830	519	416	623		416	830	168	168	789
Total sulfur	52	185	185	185	185	185	185	229	259	94	228	228
Total methyl	109	109	172	140	130	151		130	172	106	106	169
Total food (gm)	230	254	240	262	305	282	285	291	261	266	236	272
Mean total gain (gm, 28 days)	37.7	57.9	51.5	62.0	74.7	65.2	67.3	74.3	64.1	67.7	64.9	67.5
Gain/100 gm food (gm)	16.4	22.8	21.4	23.6	24.2	23.0	23.6	25.5	24.1	25.2	27.0	24.8
Protein efficiency [4] (gm)	2.5	3.5	3.2	3.6	3.7	3.5	3.6	3.9	3.7	3.8	4.1	3.8

[1] All casein diets contained 273 mg % of choline chloride (equivalent to approximately 88 mg % of methyl groups). Similarly, the lactalbumin diets were supplemented with 278 mg % of choline chloride. The remainder of the methyl groups was derived from the methionine in the protein and the supplementary amino acids.

[2] A combination of groups IV to VI inclusive. See text for explanation.

[3] All calculations of amino acid, sulfur, and methyl contents are expressed as milligrams per 100 gm of diet.

[4] Gain per gram of protein consumed.

plements were the same (groups XI and XII) as those used with the casein diet (groups II and III) in comparable experiments.

Mean gains per 100 gm of food consumed were calculated, and the significance of the differences was determined by applying to the data the Student-Fisher "t" test and the F test for variance (Snedecor, '46).

In table 1 are shown the mean gains in weight per 100 gm of food for the groups fed the basal diet and the diets to which the sulfur-containing amino acids were added as supplements. Total gains in weight for the 28-day period and protein efficiencies, calculated as weight gains per gram of protein consumed, are also given. Thus the values for the supplemented diets demonstrate the changes in utilization of the casein and lactalbumin for growth associated with the addition of the individual amino acids. The differences in the mean values of the gains per 100 gm of food between the various groups have been calculated, and their statistical significance is indicated in table 2.

Supplemental values of cystine and methionine

The addition of either L-cystine (group II) or DL-methionine (group III) to the basal diet was followed by improved growth. There was, however, no significant difference in the mean values of the gains per 100 gm of food consumed between the two diets which were equal in organic sulfur content (protein and amino acid sulfur) but in which the distribution of the total sulfur-containing amino acids was different. When the same level of sulfur was maintained but both the sulfur-containing amino acids were added as supplements in varying proportions (groups IV to VI), the mean gains were much greater than those of the animals fed the basal casein diet. They were not, however, significantly different from the mean gains per 100 gm of food of group II (cystine-supplemented diet). As is shown in table 1, there was no significant difference among the mean values for groups IV, V, and VI. The F test for variance in the 42 animals of these three groups

showed that they were members of a single population. The "t" value of the differences between the combined groups (group VII), which included all animals fed supplements of both the sulfur-containing amino acids and the methionine-supplemented series (group III) was 2.22, indicating that any

TABLE 2

Statistical analyses of differences in growth per 100 gm of food consumed

DIETS COMPARED	GROUP	DEGREES OF FREEDOM	"t"	P
Casein	I vs.			
casein and cystine	II	75	7.434	0.01
casein and methionine	III	70	5.238	0.01
casein and cystine and methionine	VII	93	9.823	0.01
lactalbumin	X	89	11.786	0.01
Casein and methionine	III vs.			
casein and cystine	II	41	1.315	
casein and cystine and methionine	VII	59	2.219	0.02–0.05
casein + 0.6% methionine + 0.25% cystine	IX	28	2.079	0.02–0.05
Casein and cystine	II vs.			
casein and cystine and methionine	VII	63	0.85	
casein + 0.5% cystine + 0.2% methionine	VIII	34	2.422	0.02–0.05
Lactalbumin	X vs.			
lactalbumin and cystine	XI	53	1.963	
lactalbumin and methionine	XII	39	0.160	
Casein + 0.5% cystine + 0.2% methionine	VIII vs.			
casein and 0.6% methionine + 0.25% cystine	IX	21	1.193	
casein and cystine and methionine	VII	52	1.722	
lactalbumin and cystine	XI	27	1.550	
Casein + 0.6% methionine + 0.25% cystine	IX vs.			
casein and cystine and methionine	VII	51	0.463	
lactalbumin and methionine	XII	12	0.259	

superiority of the combined amino acid supplements was of marginal significance.

A series of experiments (groups VIII and IX) was designed to determine the effect of a *higher total sulfur level* in the diet by increasing the amounts of the two supplementary sulfur-

containing amino acids beyond the levels fed groups IV to VI. Group VIII is to be compared with group II, since the diet of the former group contained supplemental methionine (0.2%); i.e., more than that in the diet of group II. The total methionine level was 0.416%, a value below the "minimum" methionine requirement of rats fed a mixture of amino acids which contained no cystine (Womack and Rose, '41). Similarly, groups III and IX may be compared, since the diet of the latter group differed from that of the former only in the incorporation of added cystine. The amount of sulfur (total protein and amino acid) was higher (259 mg) than that in any of the diets of the present study; 0.83% methionine was present, as in the diet of group III, but, in addition, 0.25% of cystine was added to give a total cystine content of 277 mg %. The increased amounts of supplementary sulfur-containing amino acids in varying ratios did not influence the gain per 100 gm of food consumed nor the mean total gain (compare groups IV, V, VI, VIII and IX). Gains per 100 gm of food were similar for groups IV to VI (185 mg % of sulfur) and groups VIII and IX (229 and 259 mg % of sulfur, respectively); i.e., the addition of methionine so that the total methionine content of the diet was nearly equal to (group VIII), or in considerable excess of (group IX), the minimum value of Womack and Rose ('41) did not significantly change the protein efficiency of the diet.

We have also included in the present series certain experiments with commercial lactalbumin, since it is known that this mixture of milk proteins has a distribution of sulfur-containing amino acids different from that of casein (Brand, '46; Williamson, '44). Thus the basal (7.5%) casein diet used by us was estimated to contain 52 mg % of total protein sulfur, 27 mg % of cystine, and 209 mg % of methionine, while in the lactalbumin diet of equal nitrogen content the amounts of total protein sulfur, cystine, and methionine were approximately 94, 218 and 168 mg %, respectively. Rats on this latter diet (group X) showed growth superior to that of rats on the

basal casein diet (group I) and equal to that observed when the basal casein diet was supplemented with either cystine, methionine or mixtures of these two amino acids in any of the proportions studied. These results were obtained even though the methionine content of the lactalbumin diet was estimated to be less than that of any other diet of the present series. In contrast to similar experiments with the basal casein diet (group I), equivalent in its content of total nitrogen, it was not possible under the conditions of our experiments to demonstrate any increase in the rate of growth when either cystine (group XI) or methionine (group XII) was added as a supplement to the lactalbumin. The total sulfur of these diets was comparable to that of the diet of group VIII but the sulfur distribution was different. The diet of group XI contained much more cystine than did any other diet of our series.

Our chief concern in the present study has been the control of non-protein dietary factors so that any differences observed could be ascribed to the cystine or methionine supplements added to the low protein diet. One such dietary factor is choline. Salmon ('47) showed that with diets containing 18% or less of casein, the limiting factor was the dietary methyl groups. If adequate vitamins and fat were present, the addition of a non-protein source of labile methyl groups (e.g., choline) resulted in an increased rate of growth and afforded protection from liver and kidney damage. Experiments by Treadwell ('45) also demonstrated that the chief dietary defect of an 18% protein diet (15% casein and 3% arachin) was an inadequate supply of methyl groups.

In the earlier work of Womack, Kemmerer and Rose ('37), in which no cystine was included in the mixture of amino acids which supplied the nitrogenous portion of the diet, a minimum level of 0.6% of dietary methionine was established as essential for good growth. The partial replacement of methionine by cystine was possible only to a limited degree; one-sixth of the basal requirement for methionine could be met by cystine. Since the content of choline (and methyl

groups) in the vitamin concentrate used was not known, it is not possible to estimate the total methyl groups present in this diet. However, it seems probable that the diet, which supplied 0.6% methionine, the lowest level to afford good growth, may have equaled or exceeded the level of methyl groups commonly held to be minimum. Womack and Rose ('41) studied growth on other levels of dietary cystine and methionine. Growth on a diet which supplied 0.3% DL-methionine and 0.5% L-cystine was superior to that obtained with 0.3% DL-methionine alone but definitely less than the growth obtained with 1.4% DL-methionine. It is difficult to evaluate these experiments, since the total content of dietary methyl groups is unknown. It is possible that better growth might have been possible on lower levels of methionine or methionine plus cystine if a more abundant supply of methyl groups had been available.

Methionine may be important in the growth of the young white rat as (1) a source of methyl groups, (2) an amino acid essential for the synthesis of new protein, and (3) a source of sulfur for the synthesis of cystine and similar sulfur compounds. We have attempted to eliminate or minimize the need for methionine for the reactions of transmethylation by the inclusion of choline in our diets. The minimum amount of methionine in our casein diets was approximately 0.2%. The fact that good growth was obtained when either cystine or methionine was added to the basal casein diet suggests that the amount of methionine in the basal diet met or possibly exceeded the need for methionine as such for the synthesis of body protein. The supplementary value of cystine for the basal casein diet would indicate that, under the conditions of our experiments, the cystine requirements may exceed those for methionine. This may be true particularly in the young animal, in which growth of hair (and hence the requirement for cystine) is of special importance. Our experiments fail to prove, however, that greater physiological economy is achieved by the use of cystine preformed in the diet rather than cystine synthesized from methionine.

Thus equal increments in weight were observed on a dietary level of 185 mg % of total sulfur (groups II to VI) when the sulfur present as cystine ranged from less than 4% (group III) to more than 75% (group II) of the total organic sulfur of the diet. An increase of the total organic sulfur of the diet to 229 mg % (group VIII) and 259 mg % (group IX) did not result in superior growth in comparison with that of the groups receiving 185 mg % of sulfur with variable distributions of the sulfur-containing amino acids. It may also be observed that with none of our diets did we note any toxic effects of methionine (Van Pilsum and Berg, '50; Wretlind, '50). It should be pointed out, however, that the highest level of dietary DL-methionine in these experiments was 830 mg % and that a considerable part of the sulfur-containing amino acids was present in combination in the protein molecule and not in free condition.

After the experimental work here discussed had been completed and the results prepared for publication, Wretlind and Rose ('50) presented data concerning the requirements for the sulfur-containing amino acids in which the experimental conditions varied from those on which the original observations of Womack et al. ('37, '41) were based. Cystine (0.2%) was uniformly present in the amino acid mixture fed, choline (0.2%) was included in all diets, and a mixture of synthetic vitamins was fed. Under these conditions, slight differences (statistically not significant) were observed in the mean gains with concentrations of methionine varying from 0.3 to 0.8%. These workers propose 0.4% of methionine as the minimum level for adequate nutrition of the young white rat. It seems probable in the light of our own experiments that the presence of cystine in the amino acid mixture fed, and of choline, may have influenced the requirement for methionine. However, it is not possible to make a direct comparison with our own data, since the experimental conditions were so different. In confirmation of earlier work, D-methionine was utilized for purposes of growth as effectively as the L-isomer.

SUMMARY

1. Varying amounts of L-cystine, DL-methionine, and these two sulfur-containing amino acids in mixture, were added to the diets of young white rats as supplements to a 7.5% casein diet (basal diet) which contained approximately 0.2% methionine and 0.03% cystine. Choline (as the hydrochloride) was added to all diets to provide a source of non-protein methyl groups.

2. A significant increase in the rate of growth was observed when cystine was added to the basal diet, despite the fact that the methionine content of this diet was considerably lower than the amount usually accepted as adequate. Increases in the rate of growth comparable to those obtained with the cystine supplement were observed when supplements of methionine or of mixtures of cystine and methionine in varying proportions were added to the basal diet. Essentially the same growth responses with diets of equal sulfur content were observed even though the cystine sulfur ranged from 4 to 75% of the total organic sulfur.

3. The supplementary value of cystine for the basal casein diet would indicate that, under the conditions of these experiments, the cystine requirements may exceed those for methionine. These requirements may be filled by cystine present in the diet or by cystine synthesized from dietary methionine. The data do not indicate that greater physiological economy is achieved by inclusion in the diet of cystine itself rather than methionine from which cystine may be synthesized.

4. Under the conditions of the present experiments, commercial lactalbumin was more effective in promoting the growth of young white rats than was casein (7.5%) in equivalent amounts (nitrogen content), despite the fact that the methionine content of the diet used in this study was less than that of any other diet in the series. In contrast to the results with the basal (7.5%) casein diet, it was not possible to increase significantly the rate of growth when lactalbumin was supplemented with DL-methionine or L-cystine.

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EFFECT OF STANNOUS FLUORIDE IN FOOD AND IN DRINKING WATER ON CARIES PREVENTION IN RATS ON HIGH SUCROSE AND COARSE CORN DIETS[1, 2]

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It has been demonstrated that stannous fluoride is more effective than sodium fluoride *in vitro* for decreasing the acid solubility of powdered enamel and dentin (Muhler and Van Huysen, '47; Manly and Bibby, '49; Muhler, Boyd and Van Huysen, '50). Also, it has been shown that stannous fluoride in concentrations up to 10 parts of fluorine per million in the drinking water is superior to stannous chloride or stoichiometric amounts of fluorine as sodium fluoride in reducing the incidence and severity of carious lesions produced by feeding a diet containing a large percentage of coarsely ground corn (Muhler and Day, '50). This suggests that more attention should be given to the caries-preventive effect of different sources of fluorine, and that use of stannous fluoride may be advantageous in attempts to lower the incidence of human dental caries. Thus, the major objective of this investigation was to compare the effectiveness of stannous fluoride with that of sodium fluoride when ingested in the food as well as in the drinking water.

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[2] Contribution No. 517 from the Department of Chemistry, Indiana University.

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EXPERIMENTAL

Weanling rats [4] were divided as uniformly as possible according to weight and sex to make up the three different series. Series I consisted of rats restricted to a modified Hopper, Webber and Canniff ('32) diet containing 64% coarsely ground corn. The fluoride supplements used were furnished in the drinking water. Series II were given the same diet but the fluoride supplements were added to the food. Series III animals were given a purified diet high in sucrose and the fluoride supplements were added to the drinking water.

The composition of the diet high in corn (diet I) has been described previously (Muhler and Day, '50). The diet high in sucrose (diet II) was composed, in per cent, of casein ("vitamin-free") 18, sucrose 62, salts (Jones and Foster, '42) 4, vitamin mixture 1, cottonseed oil [5] 15, and fish liver oil (oleum percomorphum) 20 drops per kilogram of diet. The vitamin mixture contained thiamine hydrochloride 5 mg, riboflavin 5 mg, nicotinic acid 25 mg, pyridoxine hydrochloride 5 mg, calcium pantothenate 25 mg, inositol 50 mg, *p*-aminobenzoic acid 50 mg, folacin 2 mg, biotin 0.1 mg, choline chloride 6 gm, and sucrose 43.8 gm.

The fluoride supplements for the series II animals were mixed thoroughly with a small portion of the basal diet. This was next added to the rest of the diet and mixed with it by means of a mechanical mixer. Chemical analyses (Tarbell, Voss and Smith, '49) of several different samples from various batches of the supplemented diets showed that the fluorine concentration was uniform.

Stock supplements for the drinking water given to animals in series I and III were made up approximately once every two weeks. The desired dilutions were made up with such frequency that none were more than three days old when they were given to the animals. Even so, the stannous fluoride preparations underwent considerable hydrolysis with the

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[5] Wesson.

formation of a precipitate. Frequent chemical analyses of the clear supernatant solutions showed that the fluorine concentration was not lowered and that it remained constant. The glass containers did not become etched. Because stannous salts readily change to stannic, it is highly probable that most of the tin compounds used had undergone such oxidation before being ingested by the animals. Nevertheless, the tin compounds used in this investigation will be referred to here as stannous compounds.

Food and water were furnished ad libitum. In all cases the supplements were started at the time the animals were restricted to the experimental diets. Administration of the supplements was continued 140 days, at which time the rats were killed. The method of preparing the heads for examination and of counting the carious lesions was identical to that reported previously (Muhler and Day, '50).

Briefly, the method of counting was as follows: Each cavity which involved only the enamel was counted as a single lesion. However, if there was extensive destruction of the tooth, the score was three lesions in the case of the first molars, two lesions in the case of the second molars, and only one lesion in the case of the third molars. This procedure was adopted because the first, second, and third molars have, respectively, three, two and one fissure each. Under these experimental conditions the lesions are initiated in such fissures. In order to make satisfactory allowance for tooth destruction involving more than one initial carious fissure per tooth, this has been found to be a dependable method (Muhler and Day, '50).

RESULTS AND DISCUSSION

The principal results are summarized in table 1. In accord with previous findings (Muhler and Day, '50) based on lower concentrations of fluorine, stannous fluoride in the drinking water was decidedly more effective than sodium fluoride in decreasing the incidence and severity of carious lesions. For example, in 53 rats given 18 p.p.m. of fluorine as stannous fluoride in the drinking water (series I) the number of carious

lesions was somewhat less than two per rat, but in 51 animals given 21 p.p.m. of fluorine as sodium fluoride the average number was slightly less than 4 per rat. Both forms of fluorine had a protective effect, because 74 unsupplemented controls developed approximately 8 lesions per rat.

Earlier observations (Muhler and Day, '50) indicated that tin as a stannous chloride has some protective effect. The more extensive data presented here give additional support for that conclusion. In these experiments the amount of tin in the drinking water was approximately 50 p.p.m. (series I) and the amount in the diet was about 10 times greater (series II). There is some possibility that the effect is due to the small amount of fluorine in the stannous chloride, because the degree of protection is about the same as that in rats given 2 p.p.m. of fluorine as stannous fluoride in the drinking water (Muhler and Day. '50).

The stannous fluoride is also superior to sodium fluoride when the dry salts are mixed with the diet containing coarsely ground corn (series II). At two different levels of stannous fluoride in the diet — i.e., 26 p.p.m. and 44 p.p.m. of fluorine — the number of carious lesions was approximately two per rat. In contrast, supplements of sodium fluoride, at levels furnishing 22 p.p.m. and 51 p.p.m. of fluorine respectively, resulted in a caries incidence which was about twice as high. The number of lesions was less than one per rat in the case of animals receiving the diet with 159 p.p.m. of fluorine as stannous fluoride. Thus the protective effect, as determined by this method, increases with increasing amounts of stannous fluoride. The degree of protection seemed to be as great, if not greater, per unit amount of fluorine when the fluorides were administered in the food. For example, for rats receiving 18 p.p.m. of fluorine as stannous fluoride in the drinking water, it is estimated that the daily fluorine intake was about 1 mg; but for rats receiving 26 p.p.m. of fluorine as stannous fluoride in the diet, the fluorine intake may not have been more than 0.5 mg per day.

TABLE 1

Caries-preventive effect of stannous fluoride, stannous chloride and sodium fluoride in the drinking water as compared with the effect in the diet

SUPPLEMENT	SEX	FLUORINE IN		NO. OF RATS	WEIGHT GAIN	NO. OF LESIONS	DECREASE
		Water	Diet				
		p.p.m.	*p.p.m.*		*gm*		%
			Series I (corn diet)				
Stannous fluoride [1]	M	18	8	34	264	1.2 ± 0.60 [2]	85
	F	18	8	19	166	2.2 ± 0.85	71
Sodium fluoride	M	21	8	30	249	3.4 ± 0.97	58
	F	21	8	21	161	4.0 ± 0.80	47
Stannous chloride [3]	M	1	8	21	250	4.8 ± 1.19	41
	F	1	8	21	158	4.0 ± 1.14	47
None	M	0.3	8	44	243	8.1 ± 0.83	..
	F	0.3	8	30	163	7.5 ± 0.66	..
			Series II (corn diet)				
Stannous fluoride [4]	M	0.3	26	22	299	2.2 ± 0.94	59
	F	0.3	26	16	180	2.4 ± 0.85	63
Stannous fluoride [4]	M	0.3	44	20	261	2.0 ± 0.60	62
	F	0.3	44	22	160	1.8 ± 0.53	72
Stannous fluoride [4]	M	0.3	159	22	92	0.5 ± 0.36	91
	F	0.3	159	16	113	0.9 ± 0.30	83
Sodium fluoride	M	0.3	22	22	313	4.3 ± 0.75	18
	F	0.3	22	22	166	5.2 ± 0.57	20
Sodium fluoride	M	0.3	51	22	246	3.0 ± 1.11	43
	F	0.3	51	22	163	4.2 ± 1.00	35
Stannous chloride [5]	M	0.3	9	20	209	4.0 ± 0.95	25
	F	0.3	9	22	161	4.7 ± 0.95	28
None	M	0.3	8	20	316	5.3 ± 1.10	..
	F	0.3	8	28	172	6.5 ± 0.94	..
			Series III (high sucrose diet)				
Stannous fluoride [1]	M	9	2	18	285	1.2 ± 0.23	65
	F	9	2	20	142	1.4 ± 0.97	61
None	M	0.3	2	20	243	3.4 ± 0.40	..
	F	0.3	2	14	137	3.6 ± 1.18	

[1] Obtained from the Harshaw Chemical Co., Cleveland, Ohio.

[2] Standard error of the mean.

[3] The concentration of tin in the water was approximately 50 p.p.m.

[4] Obtained from the A. D. McKay Co., New York, N. Y.

[5] The amount fed to both sexes furnished 500 p.p.m. tin. This was stoichiometrically equivalent to the tin in stannous fluoride fed at a level furnishing 159 p.p.m. of fluorine.

Almost from the beginning of the series II experiment it was obvious that stannous fluoride is toxic at the level of 159 p.p.m. of fluorine in the diet. Growth was retarded and the animals were unthrifty in appearance and less active. At this level of fluorine the concentration of tin was approximately 500 p.p.m. The toxicity may have been due, in part, to the large amount of tin. This is probable because rats given a stoichiometric amount of tin, as stannous chloride, failed to gain as much weight as the controls. However, the data clearly show that stannous fluoride has a marked effect on the incidence of caries at concentrations far below those which are injurious. Except in those rats given the excessive amounts of stannous fluoride and stannous chloride indicated, there were no evidences of health impairment.

The male rats given the largest amount of stannous fluoride (159 p.p.m. of fluorine) suffered a greater degree of growth impairment than the females on the same diet. The gain in weight by the males was only approximately 30% as much as that of their non-supplemented controls, but the gain of the females was 65% of that of their female controls. This apparent sexual difference also occurred with the rats fed large amounts of stannous chloride.

It was of interest to test the effect of stannous fluoride on the prevention of caries in rats on another type of diet, one high in sucrose and free from fermentable particles likely to be lodged in the occlusial surfaces of the teeth. Also, such a high sucrose diet would not induce fracturing of cusps as in the case of the coarsely ground corn diet. The high sucrose diet selected (diet II) was only approximately one-half as effective as the coarsely ground corn diet in producing dental caries. However, the data (series III) show that 9 p.p.m. of fluorine, as stannous fluoride, in drinking water was accompanied by a marked decrease in the incidence of caries. Thus it is apparent that stannous fluoride decreases the incidence of dental caries produced by two different experimental methods.

We have considered that the greater caries-preventive effect of stannous fluoride when it is administered in drinking water may be due to the low pH of the solution. The pH is approximately 3.0 in solutions containing 10 p.p.m. of fluorine as stannous fluoride, but in equivalent concentrations of sodium fluoride the pH is near 4.5. If this should be an important factor, stannous fluoride ought to have substantially no superiority over sodium fluoride when it is mixed with the dry corn diet (diet I). Because stannous fluoride is more effective than sodium fluoride under these conditions, it is possible that the pH of the fluoride solution in the mouth is not the primary determinant of differences in caries-preventive action. This question is under investigation in our laboratory.

In the case of rats on diet I receiving stannous fluoride, either in the drinking water or in the food, the severity of the caries was less than in animals given equivalent amounts of fluorine as sodium fluoride. The cavitations were not as wide or deep and they did not involve more than their own dependent fissures. In the case of lesions in rats receiving sodium fluoride or stannous chloride, there was occasionally a complete loss of at least one whole crown, and in some animals more than one crown was completely destroyed by cavitation. Unsupplemented control animals on diet I showed many instances of complete whole crown destruction, which is in accordance with previous experience in this laboratory and elsewhere.

It is of interest to consider the effect of the fluorides on the incidence of depigmentation of the anterior teeth. That the ingestion of large amounts of fluoride results in such depigmentation is well established (Pindborg et al., '46). Loss of pigment did not occur in any of the animals given the supplements in the drinking water. It occurred only in the rats given sodium fluoride at the level of 51 p.p.m. of fluorine in the diet and stannous fluoride at 159 p.p.m. of fluorine. Depigmented anterior teeth were found in all the animals of the latter group and in all the males of the former. However, only 18% of the females of the sodium fluoride group had

depigmented teeth. Conceivably this apparent sexual difference is related to the differences between males and females in the amount of food ingested, and therefore in the amount of fluorine obtained.

The depigmentation was paralleled by the tendency of one or more anterior teeth to be fractured. This impaired the normal mechanism of attrition by which the length of these teeth, which grow continuously, is kept constant. As a result, the characteristic overgrowth of the unopposed and intact incisors became most pronounced. The pattern resembled that described originally by McCollum et al. ('25).

SUMMARY

A total of 588 weanling rats was used to determine the effect of different amounts of stannous fluoride and sodium fluoride on the incidence of experimental dental caries.

Stannous fluoride furnishing 18 p.p.m. of fluorine in the drinking water decreased the incidence of caries appproximately 75% in rats on a diet high in coarsely ground corn, whereas the decrease was about 50% when the supplement was sodium fluoride furnishing 21 p.p.m. of fluorine. Also, stannous fluoride furnishing 9 p.p.m. of fluorine in the drinking water, greatly lowered the incidence of caries in rats fed a high sucrose diet.

In addition, different amounts of stannous fluoride and sodium fluoride were added to the high corn diet. In all cases the stannous fluoride was superior to the sodium fluoride in preventing caries.

Stannous flouride furnishing 159 p.p.m. of fluorine and 500 p.p.m. of tin in the food was definitely injurious to the rats, as indicated by the marked impairment of growth and the abject appearance of the animals. However, this compound appeared to be no more toxic than sodium fluoride. With concentrations no higher than 26 p.p.m. of fluorine in the food or drinking water there was no evidence of toxicity.

The reason for the superior effect of stannous fluoride remains to be determined. The tin moiety alone may have

some beneficial effect, because large amounts of stannous chloride decreased the number of carious lesions, whether it was fed or given in the drinking water.

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SOME EFFECTS OF CALORIC INTAKE ON NITROGEN BALANCE IN DOGS [1]

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FOUR FIGURES

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Numerous studies have demonstrated that retention of nitrogen in the animal is in part a function of the caloric intake. This function has been emphasized by Allison et al. ('45, '46), who demonstrated in the dog that urinary nitrogen excretion increased as the caloric intake decreased, but that the nitrogen balance index of the dietary protein was not altered if the calories amounted to 50% or more of the normal requirement. Bosshardt et al. ('46) pointed out that these results suggest two responses to a systematic reduction in calories. The first response involves catabolism of nitrogenous constituents without an alteration in the index, while the second response, associated with a severe caloric restriction, is correlated with a marked decrease in the nitrogen balance index. This second response has been reported in the mouse (Bosshardt et al., '46, '48), in the rat (Bosshardt et al., '46; Stevenson et al., '46; Willman et al., '47; Benditt et al., 48), in the dog (Allison et al., '46) and in man (Schwim-

[1] This paper reports research undertaken, in part, in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number *323* in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of the Army.

Some of these data were presented at the American Chemical Society meeting, Atlantic City, New Jersey, 1949.

mer and McGavack, '48; Johnson et al., '47). The first response may be the result of increased catabolism of a portion of the nitrogenous constituents of the metabolic pool. The second response could be associated with a shift in catabolism following partial depletion of the pool. Unpublished data obtained on the dog suggest that the nitrogen balance index is not reduced in the presence of restricted caloric intakes until the protein and fat stores have been depleted to some extent. Some dogs resisted the depleting effects of a restricted caloric intake much more than others. The following studies were planned, therefore, to analyze responses to caloric restriction in more detail and to compare the resistances of different dogs to the effects of reduced calories. This analysis is limited to data obtained on dogs fed a diet low in fat but containing sufficient casein nitrogen to maintain nitrogen equilibrium in an average animal.

TABLE 1

Basic diet

INGREDIENTS	CALORIES	WEIGHT	VITAMINS	AMOUNT
	%	gm		mg/day/kg body wt.
Casein (vitamin-free)	5.36	5.35	Thiamine	0.03
Sucrose	20.00	19.95	Riboflavin	0.025
Dextrose	35.94	35.86	Nicotinic acid	0.250
Dextrin	33.70	33.60	Calcium pantothenate	0.200
Lard	5.00	2.24	Pyridoxine	0.015
Salt mixture	..	1.20	Choline	15.0
Agar	..	1.80	2-Methyl-naphthoquinone	0.00001
Water	..	140.00	Calcium tocopherol monosuccinate	0.5
			Biotin	0.01
			Folic acid	0.008
			Vitamin A	470 units
			Vitamin D	85 units

METHODS

Adult fox terriers were used in these studies. The basic experimental diet is recorded in table 1.

The calories were increased or decreased in this diet by adding or removing carbohydrate. The fat content was kept at 5% of the calories and the protein intake was kept constant. The per cent of protein increased, therefore, as the caloric intake was reduced. All diets were fed on the basis of Cal./day/m^2 of probable body surface (Cowgill, '28). Nitrogen balance indexes were determined as described previously (Allison et al., '49). Urea and ammonia nitrogen were analyzed by the method of Conway ('33).

TABLE 2

Average values for caloric intake, nitrogen intake, nitrogen output and nitrogen balance for three dogs

CALORIES/DAY/M^2 OF BODY SURFACE	NITROGEN INTAKE	URINARY PLUS FECES NITROGEN	NITROGEN BALANCE
	gm/day/m^2	*gm/day/m^2*	
3,190	3.82	3.14	+ 0.68 ± 0.16 [1]
2,860	3.82	3.64	+ 0.18 ± 0.14
2,290	3.81	3.68	+ 0.13 ± 0.08
1,910	3.82	3.92	— 0.10 ± 0.01
1,530	3.82	3.91	— 0.09 ± 0.06
960	3.81	3.84	— 0.03 ± 0.07
480	3.85	4.07	— 0.22 ± 0.35
95	3.90	4.64	— 0.74 ± 0.42

[1] Standard error (6 determinations).

RESULTS

The data in table 2 illustrate the change in nitrogen balance which is associated with a decrease in caloric intake from 3,190 to 95 Cal./day/m^2 of body surface. The lowest caloric intake compatible with nitrogen equilibrium under these experimental conditions was approximately 2,290 Cal./day/m^2, a relatively high requirement for dogs but found in small active animals such as the fox terrier.

Each dog received a constant nitrogen intake of 3.82 gm of nitrogen/day/m^2, sufficient to maintain nitrogen balance in the presence of adequate caloric intakes. Each nitrogen balance was determined during an 8-day feeding period, short enough to prevent marked loss or gain in nitrogen in the body

of the animal. As the number of calories were reduced there was a significant decrease in nitrogen balance, the dogs being shifted to negative nitrogen balance. That this increased excretion of nitrogen did not alter the nitrogen balance index of the dietary protein, an index which is a function of the amount of dietary nitrogen retained in the body of the animal, is illustrated by the data in table 3.

TABLE 3

The effect of caloric intake on the nitrogen balance index of casein

CALORIES/ DAY/M^2 OF BODY SURFACE	NO. OF DETER-MINA-TIONS	ABSORBED NITROGEN	NITROGEN BALANCE	PROTEIN-FREE NITROGEN BALANCE	DIGESTI-BILITY	NITROGEN BALANCE INDEX
		gm/day/m²	*gm/day/m²*	*gm/day/m²*	%	
2,860	3	3.78	+ 0.64	— 2.14	95 ± 0.34	0.74 ± 0.04 [1]
1,680	14	3.23	+ 0.19	— 2.17	100 ± 0.46	0.74 ± 0.01
670	12	2.58	— 1.44	— 3.32	99 ± 0.22	0.73 ± 0.05
480	6	2.84	— 1.39	— 3.38	99 ± 0.67	0.70 ± 0.06

[1] Standard error.

Thus the first response to caloric restriction was an increase in the excretion of nitrogen without a reduction in the nitrogen balance index of the casein. Further evidence for the regular increase in excretion of nitrogen when the caloric intake but not the protein intake is reduced is illustrated in figure 1, where urinary nitrogen and urea nitrogen are plotted against calories. In other series of experiments urinary ammonia nitrogen has been shown to decrease with reduction of caloric intake (see fig. 2), a decrease which may be the result of an alteration in acid base balance. Feces nitrogen also decreased with caloric intake, a decrease which was independent of digestibility and, therefore, may be associated with a reduction in bacterial nitrogen or body nitrogen excretion, or both.

The animal does not remain static on a restricted caloric intake, but rather drifts into a condition where nitrogen retention is markedly reduced. The data in figure 3 illustrate this drift in three dogs fed 3.82 gm of casein nitrogen/day/m^2

and 478 Cal./day/m² of body surface. These three dogs were chosen because of a difference in protein stores. The stores of dog A were relatively full, hence the nitrogen balance was negative in the presence of the amount of casein nitrogen fed. The negative balance resulted in some depletion and a drift

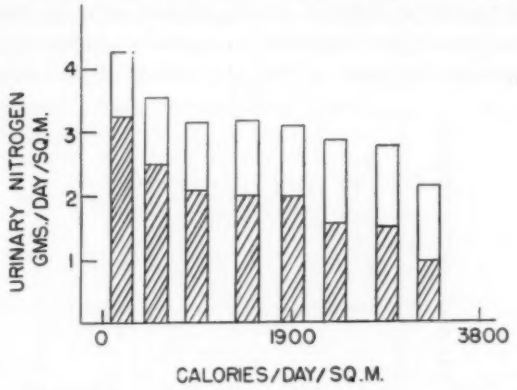

Fig. 1 Average urinary nitrogen (gm/day/m²) versus caloric intake (Cal./day/m² of body surface) of three dogs fed 3.82 gm casein nitrogen/day/m². The slanted bars represent urea nitrogen; the white bars represent all other urinary nitrogen.

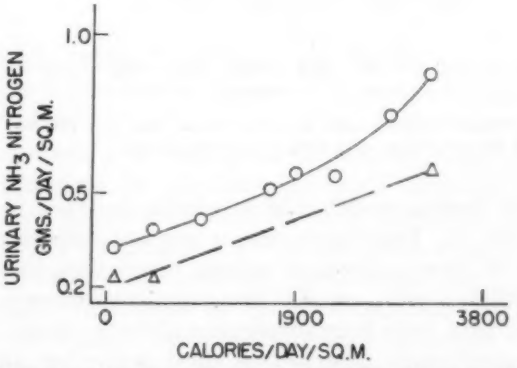

Fig. 2 Average urinary ammonia nitrogen (gm/day/m²) versus caloric intake (Cal./day/m² of body surface). The ○ represent ammonia nitrogen of three dogs fed 3.82 gm. casein nitrogen/day/m². The △ represent ammonia nitrogen of three dogs fed a protein-free diet.

toward positive balance. Before nitrogen equilibrium was reached, however, the gradual drift toward negative balance associated with a marked caloric restriction began to take place. The stores of dog B were lower than those of dog A, resulting in an initial nitrogen equilibrium. The gradual drift into negative balance is illustrated also by these data. Dog C was somewhat depleted in proteins, hence this animal was in positive balance at the beginning of the experiment.

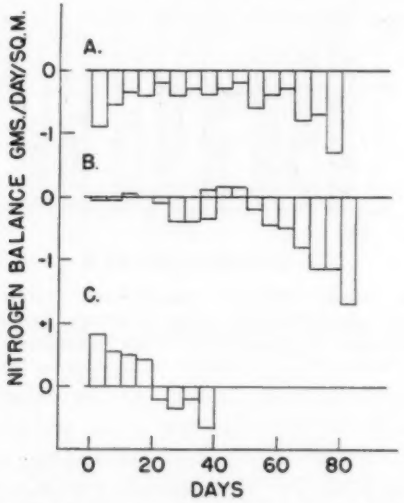

Fig. 3 Nitrogen balance (gm/day/m²) versus days fed 480 Cal./day/m² of body surface for three dogs with different protein stores.

Continued feeding resulted in a regular decrease in the balance in dog C. Thus there was a marked difference in the response of these animals to caloric restriction, the depleted animal drifting most rapidly into negative nitrogen balance.

Similar data have been obtained with other dogs. In some of the experiments protein-free diets were fed alternately with the diets containing protein so that indexes for the protein could be calculated. The results of one of these experiments are illustrated in figure 4. The reduction in index

occurred when the animal drifted into negative nitrogen balance. The shift toward negative balance can be interpreted, therefore, as one of utilization of both body and dietary nitrogen for energy purposes, leading to marked tissue breakdown. It is obvious that one dog was capable of resisting the second response (decreasing value of the index and increasing nitrogen loss) over a longer period of time than the other two animals. Studies are under way to determine the factors in the animal which control this resistance to the stress of reduced calories.

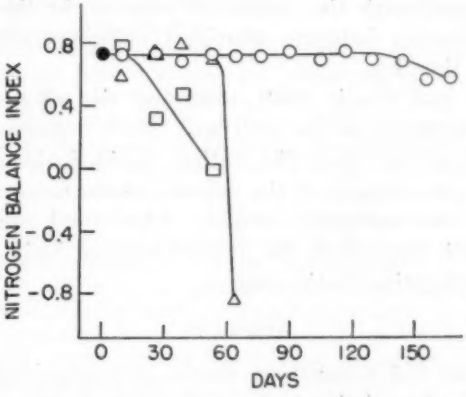

Fig. 4 Nitrogen balance index versus days fed 480 Cal./day/m² of body surface for three dogs, showing the variation in response to a reduced caloric intake.

The response to a restriction in calories, however, varies with the animal, its physiological state, and the diet fed. It should be emphasized that the data presented here were obtained while feeding a low fat diet containing a quantity of casein which would maintain the average dog in nitrogen equilibrium. Variations in the diet and the physiological state of the animal can markedly alter nitrogen retention. Schwimmer and McGavack ('48) found, for example, that young men fed a low nitrogen diet (3 gm egg white nitrogen/day) did not retain dietary nitrogen until the caloric intake had been increased to 1,500 Cal. If the nitrogen was

increased to 6 gm/day it was retained at the much lower caloric intake of 900 Cal. Bosshardt et al. ('48) demonstrated that an increase in caloric intake at a constant protein level improved protein utilization in the mouse, a utilization which was enhanced still further by the addition of more protein. These authors suggest that protein is one of the most important limiting factors in many states of semi-starvation. Their studies emphasize the need to evaluate the quantity and quality of protein in restricted diets. Beattie et al. ('47) found that the amount of nitrogen retained in an emaciated man increased with the intake of calories in the region of positive nitrogen balance, provided sufficient nitrogen was included in the diet.

Swanson and Clark ('50), studying the effect of fat on nitrogen excretion in low nitrogen diets, reported that the elimination of fat from the ration doubled the destruction of body tissue nitrogen if the caloric intake was restricted to one-fourth the customary intake. These and other dietary factors which may affect the preservation of body tissues are under investigation in the dog.

SUMMARY

Dogs were fed a constant casein nitrogen intake of 3.82 gm/day/m^2 of probable body surface. Calories were varied by altering dietary carbohydrate. Two responses to a restricted caloric intake were recognized. The first response, obtained with even a slight reduction in dietary calories, was an increase in the excretion of urinary nitrogen without any change in the nitrogen balance index of the dietary protein, a response which reflected an increase in the catabolism of proteins. Continued feeding of a markedly restricted intake depleted the protein stores of the body. The second response was a decrease in the nitrogen balance index associated with a marked increase in the excretion of both body and dietary nitrogen. This second response followed the first in the presence of a severe caloric restriction and was dangerous to the animal. Some animals resisted the second response

much more than others, a resistance which was correlated, at least in part, with the magnitude of the protein stores of the body. It is emphasized that responses to a restriction in calories vary with the physiological state of the animal and with the composition of the diet fed.

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EFFECT OF VARIOUS LEVELS OF CALCIUM, PHOSPHORUS AND VITAMIN D INTAKE ON BONE GROWTH

II. MINKS

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SIX FIGURES

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Smith and Barnes ('41) produced rickets in newly weaned mink kits by feeding a diet deficient in vitamin D and containing 0.06% calcium and 0.54% phosphorus. Two weeks of daily sunshine or the addition of 1.0% bone meal to the rachitic diet produced good healing, while 40 I.U. of vitamin D per day per mink gave slight bone healing.

In a previous paper (Harris et al., '51) data reported on the calcium, phosphorus and vitamin D requirements of growing foxes. In the experiment reported in the present paper, the same techniques were used to determine the calcium, phosphorus and vitamin D requirements of growing minks.

[1] Director, U. S. Fur Animal Experiment Station; formerly Agent, Fish and Wildlife Service, now Chairman, Institute of Nutrition, Utah State Agricultural College; and formerly Assistant Biologist, U. S. Fur Animal Experiment Station, respectively.

[2] At the time this work was conducted the research concerned with mink was under the Fish and Wildlife Service, United States Department of the Interior. Effective June 30, 1946, all activities pertaining to fur farming were transferred to the United States Department of Agriculture.

EXPERIMENTAL

Sixty-four female and 64 male kits were allotted by chance to the 64 treatments outlined in table 1. Kits were started on experiment at the weaning age of 50 days and killed at pelting time at an age of 216 days.

The minks were fed twice daily all the food they would eat. Food consumption records were kept. They were penned

TABLE 1

Experimental design [1] and deficiency symptoms [2]

I.U. VIT. D PER KG BODY WT.	% P	0.3				0.4				0.6				0.8			
SEX	% Ca	0.3	0.5	0.6	1.0	0.3	0.5	0.6	1.0	0.3	0.5	0.6	1.0	0.3	0.5	0.6	1.0
	Ca : P	1.0	1.7	2.0	3.3	0.75	1.2	1.5	2.5	0.5	0.83	1.0	1.7	0.38	0.62	0.75	1.2
Basal	♀	O	O	O	B	O	O	O	E	O	O	O	O	O	O	O	O
diet	♂	O	O	A	E	O	O	O	O	O	O	O	O	O	O	O	D
15	♀	O	O	O	B	O	O	O	O	O	O	O	O	O	O	O	O
	♂	O	O	O	E	A	O	A	O	O	O	O	E	O	O	O	D
50	♀	O	O	A	E	O	O	O	O	O	O	O	O	O	O	O	D
	♂	O	O	B	B	O	E	O	O	O	O	O	O	O	O	O	D
200	♀	O	O	O	B	O	O	O	A	O	O	O	O	O	O	O	O
	♂	O	O	C	E	O	O	E	O	O	O	O	O	A	O	O	D

[1] A male and a female were allotted at random to each of the 64 treatments.

[2] Letters indicate deficiency symptoms as determined by:

O Normal
A Lameness
B Lameness, crooked legs and x-ray
C Crooked legs and x-ray
D Loss of wieght
E Death

individually in raised, wire-floored pens in a colony-type house, darkened to keep out direct or reflected sunlight. At the start, sun-cured native bluegrass hay was used as bedding, but later this material was removed from the nests and dry shavings were used. Since the minks were not killed until they reached an age of 254 days, an x-ray photograph of the tibia-fibula of each mink was taken at approximately 125 days of age to note the bone development. The length of the bones, thickness of the bone wall and density of the bone were used,

along with the other data, to determine the requirements for calcium, phosphorus and vitamin D.

The diets and the weights and measurements of the humeri and fur at the end of the experiment were the same as have been described by Harris et al. ('51).

RESULTS

Composite samples of the diets were analyzed at the end of the experiment. On a dry basis the percentage levels of calcium were 0.342, 0.548, 0.622 and 1.04; of phosphorus, 0.326, 0.400, 0.600 and 0.759. The dry basal diet contained 0.82 I.U. of vitamin D per gram.

Deficiency symptoms

Some of the 128 kits evidenced a slight lameness (table 1) early in the experiment, but this condition disappeared by July 17, 43 days after the experiment started, in every animal but one, and in no case did it extend beyond August 6.

Four animals receiving the 1.0% calcium and 0.3% phosphorus diet died prior to September 1, 88 days after the experiment started. It is believed that the large amount of calcium in relation to phosphorus caused the death of these animals. Four other minks also died, but it is doubtful if the diet was the cause of death.

Near the end of the experiment 5 of the 8 animals receiving the 1.0% calcium and 0.8% phosphorus diets showed a loss of appetite accompanied by a severe decline in live weight. Post mortem examination of these animals when they were pelted failed to reveal why they lost weight.

Humeri, tibia-fibula analyses

The results of the statistical analyses on the humeri are summarized in table 2. Also the average total grams of ash, after the humeri were adjusted to a constant volume by covariance analyses, for the various levels and ratios of calcium, phosphorus and vitamin D are shown in figures 1 through 6.

After all the analyses on the humeri are considered it is apparent that the ratio of calcium to phosphorus is an important consideration in determining the requirements for calcium, phosphorus and vitamin D of growing minks. Ratios of calcium to phosphorus of from 0.75 : 1.00 to 1.7 : 1.00 appear to be satisfactory.

TABLE 2

Statistical analyses of humeri

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE			
		Data from x-ray 125 days of age		Data at 254 days of age	
		Width of wall	Length of bone	Bending of bone	Ash per cubic centimeter
		mm	mm	mm	
Phosphorus (P)	3	4.94	0.330 [1]	0.0011	0.0035
Calcium (Ca)	3	22.65 [1]	0.862 [1]	0.0011	0.0125 [1]
Vitamin D (D)	3	2.38	0.015	0.0026 [2]	0.0010
Sex (S)	1	124.03 [1]	9.191 [1]	0.2757	0.0076 [2]
P × Ca	9	6.38 [1]	0.285 [1]	0.0005	0.0060 [1]
P × D	9	3.98 [2]	0.057	0.0016	0.0014
P × S	3	3.31	0.115	0.0006	0.0011
Ca × D	9	1.36	0.075	0.0009	0.0015
Ca × S	3	5.75 [2]	0.137	0.0021	0.0002
D × S	3	1.09	0.046	0.0001	0.0009
Error	81	1.96	0.057	0.0009	0.0016
Total	127				

[1] Highly significant at P = 0.01.

[2] Significant at P = 0.05.

The minimum requirement of calcium and phosphorus for growing mink kits is 0.3% or below in the dry diet, provided the ratio of calcium to phosphorus is close to 1:1 and provided the diet contains an adequate amount of vitamin D.

The various levels of vitamin D fed seemed to have little effect on the humeri except that there was a tendency for the 200 I.U. levels of vitamin D plus that supplied in the basal diet (0.82 I.U. per gram of dry food) to produce more bent bones. We are therefore inclined to conclude that the vitamin D requirement of growing minks is less than that supplied in

the basal diet. This conclusion is valid if minks can utilize irradiated ergosterol.

The various levels and ratios of calcium, phosphorus and vitamin D that produced normal bones also produced normal pelts.

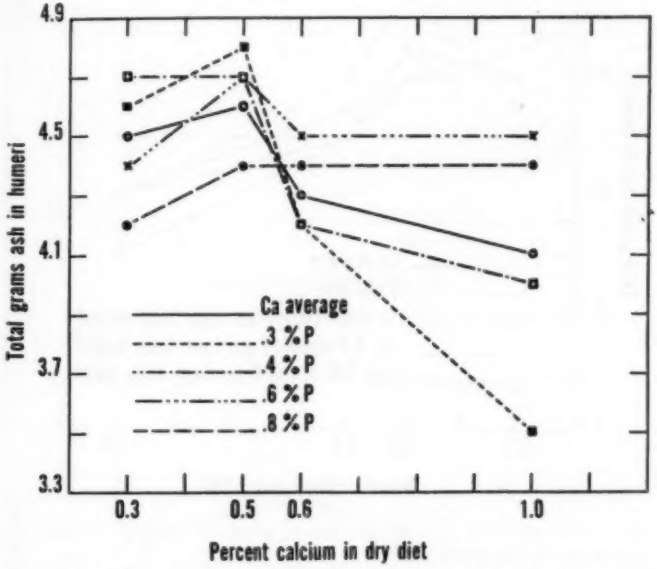

Fig. 1 The effect of calcium at various levels of phosphorus intake on the amount of ash in the humeri.

There was a tendency for the male minks to require more calcium than the female minks, but there were no other measurable differences in requirement of phosphorus or vitamin D. Male minks had larger humeri than female minks.

In practice it would seem desirable to recommend a nutrient allowance of 0.4 to 1.0% calcium and 0.4 to 0.8% phosphorus, with a calcium to phosphorus ratio of from 0.75 : 1.00 to 1.7 : 1.00. The basal diet plus 50 I.U. vitamin D per kilogram body weight did not provide more of these nutrients than the level of physiological efficiency. More calcium and phos-

phorus and less vitamin D (0.82 I.U. per gram of dry food) may produce normal bones, but our experiment did not go beyond the above levels.

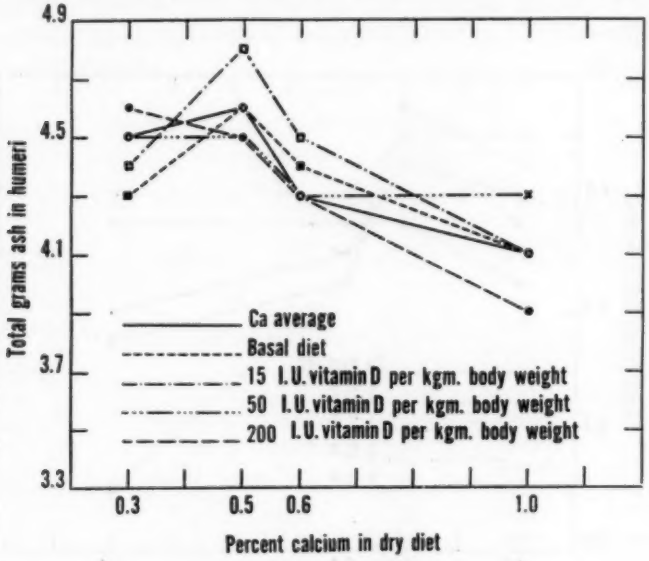

Fig. 2 The effect of calcium at various levels of vitamin D intake on the amount of ash in the humeri.

DISCUSSION

The requirements of minks for calcium and phosphorus on a percentage dry diet basis is below that of foxes (Harris et al., '51), dogs (McCay, '43), and rats (Sherman and Booker, '31). The excellent review by Mitchell and McClure ('37) gives comparative values on the calcium and phosphorus requirements of farm animals.

When the minks were fed ratios of calcium to phosphorus ranging from 0.75:1 to 1.7:1, the deficiency symptoms observed consisted of lameness and bent bones and in some cases both. The more severe deficiency symptoms, such as

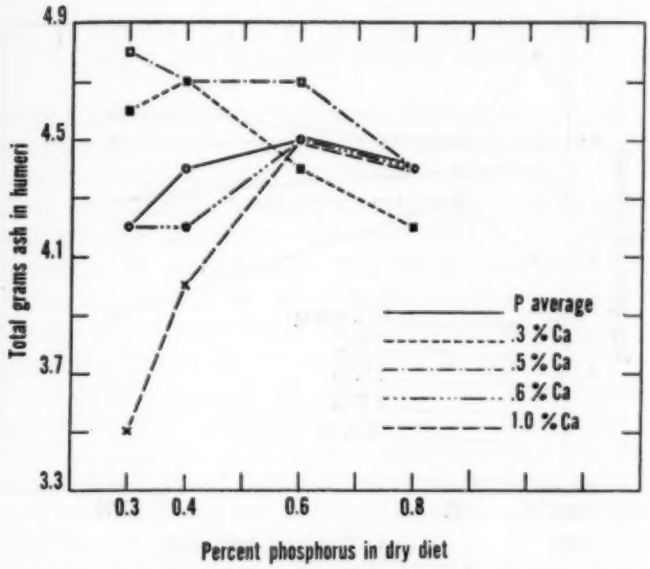

Fig. 3 The effect of phosphorus at various levels of calcium intake on the amount of ash in the humeri.

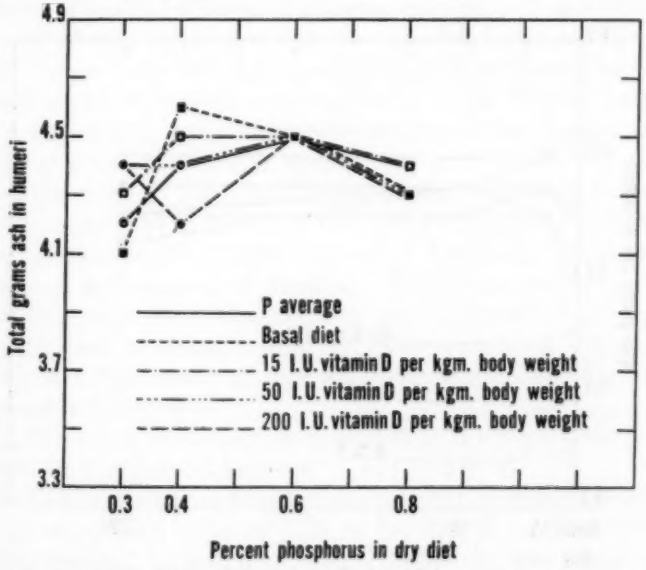

Fig. 4 The effect of phosphorus at various levels of vitamin D intake on the amount of ash in the humeri after the humeri are adjusted to an equal volume by covariance analysis.

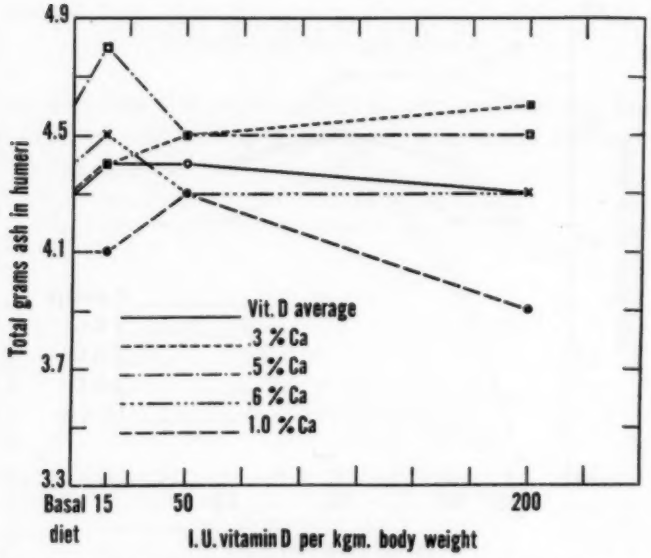

Fig. 5 The effect of vitamin D at various levels of calcium intake on the amount of ash in the humeri.

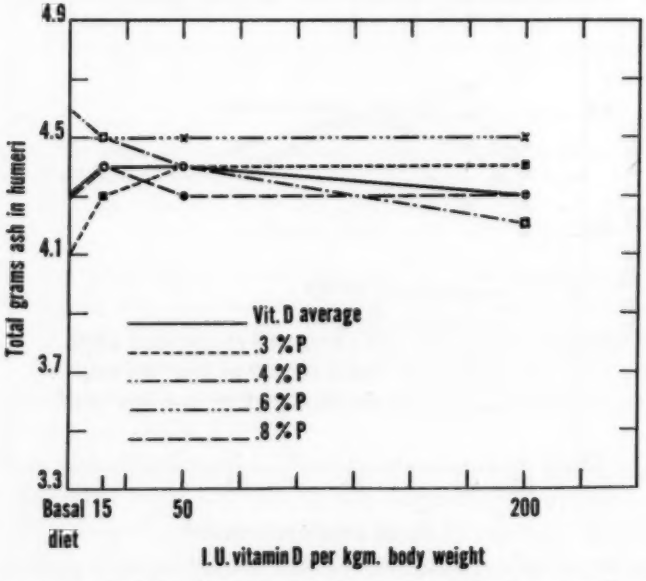

Fig. 6 The effect of vitamin D at various levels of phosphorus intake on the amount of ash in the humeri.

recurrent spasms, severely enlarged joints and cranial enlargements, which were observed in foxes (Harris et al., '51), were not observed in minks fed the same diets.

There was a slight tendency for the high level of vitamin D, 0.82 I.U. per gram dry diet plus 200 I.U. per kilogram body weight, to produce more bent bones than lower levels of vitamin D during the early stages of growth. However, at the end of the experiment the levels of vitamin D had no measurable effect on the ash content of the bones.

The ratio of calcium to phosphorus is the most important consideration in determining the requirement for calcium and phosphorus of minks. This observation emphasizes the desirability of feeding simultaneously several levels of nutrients when determining the nutrient requirements and allowances of animals.

SUMMARY AND CONCLUSIONS

In a factorially designed experiment, the effects on bone growth of each of 4 levels of calcium, phosphorus and vitamin D, and of sex, were investigated. One hundred and twenty-eight minks were fed the experimental diets, beginning at a weaning age of 50 days and continuing to the time of pelting at an age of 216 days. X-ray photographs of the humeri were taken at about 125 days of age.

At the close of the experiment the following weights and measurements were taken on the isolated humeri: weight of ash, volume, weight of ether extracted dry bone, length, bone thickness and degree of bending. Covariance analysis was used to adjust the ash content of each humerus to an equal volume and an equal weight of ether extracted dry bone. Under the conditions of this experiment the following conclusions are justified:

1. The ratio of calcium to phosphorus for a growing mink should be maintained between 0.75 : 1.00 and 1.7 : 1.0.

2. The minimum calcium and phosphorus requirement of the growing mink is below 0.3% in the dry diet. The vitamin D requirement is less than that supplied by the basal diet (0.82 I.U. per gram of dry food).

3. In practice a nutrient allowance of 0.4 to 1.0% calcium, 0.4 to 0.8% phosphorus, and 0.82 I.U. vitamin D per gram of dry food would give satisfactory results, provided the above calcium to phosphorus ratio is maintained.

ACKNOWLEDGMENTS

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THE ROLE OF VITAMIN E IN THE PRODUCTION OF NUTRITIONAL LIVER INJURY IN RATS ON LOW CASEIN DIETS [1, 2]

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The type of hepatic injury observed by Weichselbaum ('35) in rats after a few weeks on a low casein diet was recognized by Gyorgy and Goldblatt ('39) to be acute necrosis. That it differed from cirrhosis was suggested by Daft, Sebrell and Lillie ('42). Both types of hepatic injury were induced independently by Himsworth and Glynn ('44a) on diets deficient in sulfur-containing amino acids: massive hepatic necrosis appeared in some weeks, while diffuse hepatic fibrosis followed fatty infiltration of the liver and appeared in some months. Methionine and cystine prevented necrosis (Weichselbaum, '35; Himsworth and Glynn, '44b; Gyorgy and Goldblatt, '42; Hock and Fink, '43), but choline was without effect (Gyorgy and Goldblatt, '49). Fatty infiltration of the liver, which leads to diffuse hepatic fibrosis, was prevented by lipotropic factors such as choline and methionine (Best and Huntsman, '32; du Vigneaud, Cohn, Chandler, Schenck and Simmonds, '41); it was intensified by cystine (Beeston and Channon, '36; Tucker and Eckstein, '37; Earle and Victor, '41; Daft, Sebrell and Lillie, '42; Gyorgy and Goldblatt, '42).

[1] A cooperative project with the Agricultural Experiment Station of the University of Puerto Rico.

[2] A preliminary report was presented at the XVIII International Physiological Congress, Copenhagen, August 15–18, 1950.

Discordant results in the incidence of massive hepatic necrosis led to a search for contributing nutritional factors in addition to restricted dietary protein. The protective effect of certain fats appeared to be associated with the content of tocopherols (Gyorgy, '47). Schwarz ('44), Gyorgy ('47), Gyorgy and Goldblatt ('49), Hove, Copeland and Salmon ('49), Moore ('49) and others observed that α-tocopherol prevented massive hepatic necrosis. However, it did not prevent fatty infiltration of the liver and diffuse hepatic fibrosis (Himsworth, '47; Moore, '49). Muscle degeneration, typical of vitamin E deprivation, was noted by Schwarz ('44, '49) in rats with liver injury. Schwarz ('44, '49) and Himsworth and Lindan ('49) recognized the importance, in experiments of this type, of restricting initial stores of vitamin E in the organs and tissues of the rat. The existence of factors other than deficiency of the sulfur-containing amino acids and of tocopherol remained problematical, until the recent observation of Lindan and Himsworth ('50) that massive hepatic necrosis is produced in the rat by a doubly deficient diet of the sulfur-containing amino acids and of vitamin E.

An investigation of liver injury in rats deprived of vitamin E on low casein diets was prompted by observations of animals on certain diets of polished rice, red kidney beans, and casein. These diets did not contain adequate vitamin E for reproduction in the rat (Goettsch, '49). Second generation young of females that were given sufficient α-tocopherol for reproduction and lactation rapidly acquired massive hepatic necrosis on a rice-beans-casein diet of low protein content (Goettsch, '48).

The experimental diets employed in the present study contained crude casein as the source of protein. They were restricted in vitamin E content and in protein, but were adequate in other nutrients. The experimental conditions were varied according to the following factors: (1) initial stores of vitamin E in the rats; (2) initial size of animal; (3) variations in concentration of dietary casein, lard, choline and inositol; (4) addition of methionine or of cystine; and (5)

addition of α-tocopherol. The relation of α-tocopherol to fatty infiltration of the liver and diffuse hepatic fibrosis will be discussed. The results are in general agreement with those reported by Lindan and Himsworth ('50) using yeast diets.

EXPERIMENTAL

Diets and supplements

The compositions of the diets, which were prepared from current supplies every week, are given in table 1. All of the diets were low in vitamin E content. Diet 1 is typical of diets used in vitamin E experiments (Evans and Burr, '27; Goettsch and Pappenheimer, '41). Diet 2 contains the same constituents as the low protein diets 3 to 5. When α-tocopherol

TABLE 1

Composition of experimental vitamin E-low diets

CONSTITUENTS [2]	DIETS FOR BREEDING RATS [1]		ISOCALORIC LOW PROTEIN DIETS		
	1	2	3	4	5
Crude casein	26.6	20.0	7.5	8.3	10.0
Yeast, brewers' dried	16.6	...	...	...	...
Cornstarch	33.3	68.0	86.5	79.7	64.0
Salt mixture [3]	3.4	4.0	4.0	4.0	4.0
Lard	18.3	6.0	...	6.0	20.0
Cod liver oil	1.8	2.0	2.0	2.0	2.0

[1] Ten to 30 mg DL-α-tocopherol acetate given at beginning of each gestation.

[2] Synthetic vitamins added as supplements to each kilogram of diets 2 to 5: thiamine HCl, 5 mg; pyridoxine HCl, 5 mg; riboflavin, 10 mg; niacin, 20 mg; vitamin K, 5 mg; PABA, 10 mg; Ca pantothenate, 50 mg; folic acid, 4 mg; and biotin, 0.4 mg.

[3] Modified Hawk-Oser ('31) salt mixture, with $CaCO_3$ substituted for Ca citrate.

was added, it met the minimum protein requirement for growth and reproduction in several generations of rats (Goettsch, '51).

Supplements were introduced at the following concentrations: choline chloride, 0.05 to 0.2%; inositol, 0.04 to 0.2%; DL-methionine, 0.1 to 0.3%; L-cystine, 0.1 to 0.3%; and DL-α-tocopherol acetate, 2 mg per rat per week.

Procedure

The rats were descendants of Wistar albino rats brought to the School of Tropical Medicine in 1927. Breeding rats were reared and bred on the low vitamin E but adequate diets 1 and 2, which were equally suitable for the preparation of young. To permit the birth of young, the rats were given 10 mg of α-tocopherol at the beginning of the first gestation, 20 mg at the second, and 30 mg at the third. The young did not develop muscular dystrophy at the end of lactation. In this way, young with initial low stores of vitamin E were obtained.

These young rats were given diets 3 to 5 from the 21st day, at which time the body weight was approximately 35 gm.

RESULTS AND DISCUSSION

Massive hepatic necrosis in rats on low vitamin E, low casein diets

The rats on diets 3 to 5 grew poorly. Nearly all of them, 81 of 89 (91%), shown in table 2 died suddenly after 5 to 40 days. Massive hepatic necrosis was observed grossly and confirmed by microscopic examination in all animals that died. Neither convulsions (Schwarz, '49) nor pulmonary hemorrhages (Hove, Copeland and Salmon, '49) were observed. Microscopic examination disclosed early lesions of muscular dystrophy in some of the rats.

After some weeks the survivors ceased to grow and acquired the chronic muscular dystrophy typical of vitamin E deprivation in rats (Einarson and Ringsted, '38). Some of the rats were able to maintain their body weight for months in spite of dragging hind legs which impeded access to food and water. Those that survived for more than 90 days were bred. They were invariably sterile, as judged not only by the occurrence of resorption gestations and failure to sire litters, but also by the presence at death of deeply pigmented uteri and testicular degeneration. Such sterility is characteristic of

vitamin E deficiency in rats (Evans and Bishop, '23; Mason, '26; Evans and Burr, '27). There were no lesions in the liver.

It will be seen in table 2 that the incidence of massive hepatic necrosis was not influenced by variations of 0 to 20% in the lard content of the isocaloric diets. Nor did the addition of choline or inositol, or of both, prevent the disease. The ineffectiveness of choline is in accordance with the findings of Gyorgy and Goldblatt ('49).

TABLE 2

Incidence of massive hepatic necrosis in rats on low vitamin E, low casein diets varying in concentrations of lard, choline and inositol

NO. OF DIET	% DIETARY			NO. OF RATS	RATS WITH			
					Massive hepatic necrosis		No liver lesions	
	Lard	Choline chloride	Inositol		Number	Mean time of survival on diet	Number	Mean time of survival on diet
						days		*days*
3	0	0	0	7	6	25	1	138
4	6	0	0	5	4	18	1	65
5	20	0	0	17	17	17	0	
5	20	0.05	0	10	9	16	1	47
5	20	0.20	0	6	6	21	0	
3	0	0	0.04	6	6	17	0	
4	6	0	0.04	11	11	24	0	
5	20	0	0.04	6	5	26	1	147
5	20	0	0.20	4	3	11	1	52
3	0	0.05	0.04	3	2	20	1	237
4	6	0.05	0.04	4	4	22	0	
5	20	0.05	0.04	6	4	22	2	231
5	20	0.20	0.20	4	4	21	0	

It is evident that rats deprived of vitamin E on low casein diets, either (1) die in a few weeks presenting massive hepatic necrosis, with or without early lesions of muscular dystrophy, or (2) survive for a longer time, during which they cease to grow and acquire chronic muscular dystrophy; at death there are no lesions in the liver. The absence of liver lesions may be associated with a reduction in the minimum

dietary protein requirement during periods of maintenance of body weight, which was determined (Goettsch, '51) to be one-half that required for growth and reproduction.

Initial stores of vitamin E in the rat. There is great variation among rats in ability to store vitamin E in the tissues and in the requirement of vitamin E for reproduction and for the prevention of muscular dystrophy (Evans and Burr, '27; Goettsch and Pappenheimer, '41). In order to test the effect of initial stores of vitamin E, some of the young rats of breeding females on diets 1 and 2 were given a single dose of 2 mg DL-α-tocopherol acetate on the 15th day of lactation. From the 21st day they were fed diets 3 to 5. The resulting data are presented in table 3. Five of 10 rats died with lesions of massive hepatic necrosis. The discordant results among different laboratories in the production of massive hepatic necrosis presumably have been associated with the amount of tocopherols supplied during the pre-experimental period.

Initial size of the rat. Since rats with an initial body weight of 70 to 100 gm have been used frequently, young rats with low stores of vitamin E were given diet 2 until they attained a body weight of 70 to 100 gm. Before this happened, a few of them died of chronic muscular dystrophy. The rats were given diets 3 to 5 as soon as they gained the desired body weight. Among the animals with an initial body weight of 70 gm shown in table 3, 8 of 11 acquired hepatic necrosis; in the group with an initial body weight of 100 gm, three of 22 developed the disease. In both groups there was an increase in the time of survival over that of smaller animals. These observations are in agreement with those of Lindan and Himsworth ('50).

Concentration of casein. Diets of different casein concentrations were fed to 21-day-old rats with low initial stores of vitamin E. Massive hepatic necrosis appeared in all rats on the 5.8% casein diet and in two of 6 rats on 11.6% casein. The preventive effect of higher casein diets is well-known.

Addition of methionine and of cystine. Confirmatory evidence was obtained that the sulfur-containing amino acids

prevent massive hepatic necrosis (Weichselbaum, '35; Himsworth and Glynn, '44b; Gyorgy and Goldblatt, '42; Hock and Fink, '43). As is shown in table 3, the addition to diets 3 to 5 of 0.3% DL-methionine prevented liver injury in 12 of 14 rats; the addition of 0.3% L-cystine protected 13 of 16

TABLE 3

Factors influencing the incidence of massive hepatic necrosis in rats on low vitamin E, low casein diets

FACTORS	NO. OF RATS	RATS WITH			
		Massive hepatic necrosis		No liver lesions	
		No.	Mean time of survival on diet	No.	Mean time of survival on diet
			days		*days*
1. Initial stores of vitamin E					
2 mg DL-α-tocopherol acetate on 15th day of lactation	10	5	25	5	143
2. *Initial size of rat*					
body weight (gm)					
70	11	8	43	3	102
100	22	3	71	19	184
3. *Concentration of casein*[1] (%)					
5.8	8	8	11	0	
11.6	6	2	16	4	51
4. *Addition of DL-methionine* (%)					
0.1	6	5	16	1	56
0.2	5	4	15	1	50
0.3	14	2	13	12	234
5. *Addition of L-cystine* (%)					
0.1	8	8	15	0	
0.3	16	3	7	13	239

[1] Diets contained 6% lard.

rats. Lower concentrations were not as effective, and presumably higher concentrations would have protected all rats from massive hepatic necrosis.

Chronic muscular dystrophy occurred in all rats shown in table 3 that did not acquire massive hepatic necrosis. Those receiving 11.6% casein or 0.3% methionine or cystine grew

better at first than those on the low casein diets. After some weeks all rats ceased growing; they maintained their body weight for months. They were similar to the survivors shown in table 2 in appearance, behavior and the findings at death.

Brewers' yeast as source of protein. In view of the difficulty experienced by Gyorgy, Rose, Tomarelli and Goldblatt ('50) in inducing massive hepatic necrosis in rats on an American brand of brewers' yeast, it may be of interest to include data on yeast diets. Goyco and Asenjo ('51) have regularly observed massive hepatic necrosis in rats on yeast diets since 1945. The condition occurred in a high percentage of rats on diets containing 30% food yeast (*Torula Utilis*, number 3, locally grown) and 20% brewers' dried yeast.[3]

Under the given experimental conditions an incidence of 100% massive hepatic necrosis was obtained on diets in which brewers' yeast [4] was the source of protein. The diets contained 6% lard, 4% salt mixture, 2% cod liver oil, and cornstarch. They were fed to young rats with low initial stores of vitamin E. Of 6 rats given a diet containing 13% yeast, all died with typical liver lesions; of 5 rats on a diet containing 16% yeast, all died with lesions of massive hepatic necrosis.

Addition of α-tocopherol. These data are presented in table 4. Massive hepatic necrosis was prevented in all rats receiving α-tocopherol. These results are in agreement with those of other investigators (Schwarz, '44; Gyorgy, '47; Gyorgy and Goldblatt, '49; Moore, '49).

The α-tocopherol not only prevented massive hepatic necrosis but also protected the rats from muscular dystrophy, enabling them to survive for a long period of time and to reproduce. The rats on diets 3 to 5 grew slowly, attaining a body weight of approximately 175 gm at 180 days of age. They were bred and second generation animals were born. Few of the young were able to survive the period of lactation on the low protein diets, and no third generations

[3] Fleischmann, type 2019.

[4] See footnote 3.

were obtained. In the rats on the diets containing 0.3% of either methionine or cystine, there was an improvement in the rate of growth and in the reproductive performance. Second generation young were reared and bred, and third generation young were obtained. The behavior with respect to growth and reproduction was similar to that of rats on an 11.6% casein diet.

TABLE 4

Effect of α-tocopherol[1] on the nature of liver injury in rats on low vitamin E, low casein diets, with and without methionine and cystine

NO. OF DIET	% DIETARY LARD	NO. OF RATS	NUMBER OF RATS PRESENTING						
			Massive hepatic necrosis	No liver lesions				Diffuse hepatic fibrosis	
				Not fatty		Fatty			
				No.	Mean time of survival on diet	No.	Mean time of survival on diet	No.	Mean time of survival on diet
					days		*days*		*days*
Low vitamin E, low casein diets									
3	0	6	0	3	267	3	213	0	
4	6	9	0	2	136	7	233	0	
5	20	9	0	2	215	3	148	4	250
Addition of DL-*methionine, 0.3%*									
3	0	9	0	3	202	6	197	0	
4	6	5	0	2	250	2	105	1	170
5	20	3	0	2	320	1	292	0	
Addition of L-*cystine, 0.3%*									
3	0	6	0	0		3	211	3	260
4	6	3	0	0		0		3	238
5	20	9	0	2	174	4	63	3	184

[1] Two milligrams DL-α-tocopherol acetate per rat per week.

It was impossible to observe the rats until they died, as had been intended. All rats were sacrificed when the oldest ones had survived about 10 months on the diets. Although massive hepatic necrosis was not observed in any rat receiving α-tocopherol (table 4), certain changes were noted in the livers. Thus, while about one-fourth appeared normal, one-half showed very fatty livers and one-fourth presented diffuse hepatic fibrosis.

Alpha-tocopherol apparently permitted the rats on the low casein diets to survive under conditions which favored the development of fatty infiltration of the liver and resulting diffuse hepatic fibrosis. It will be seen in table 4 that the incidence of liver injury was highest with diet 5, containing 20% lard. The addition of 0.3% of methionine did not prevent fatty changes in the liver and diffuse hepatic fibrosis in all animals; presumably, higher concentrations were needed. The addition of 0.3% of cystine aggravated the intensity of the lesions, for among 18 rats receiving cystine, 7 acquired fatty livers and 9 diffuse hepatic fibrosis. These results are in agreement with the observations of others (Best and Huntsman, '32; Tucker and Eckstein, '37; Earle and Victor, '41; Daft, Sebrell and Lillie, '42; Gyorgy and Goldblatt, '42).

SUMMARY

1. With low casein diets, massive hepatic necrosis appears rapidly in young rats deprived of vitamin E. Early lesions of chronic muscular dystrophy may occur.

2. Alterations in the lard content of isocaloric diets are without effect.

3. An increase in available protein by the addition of casein or of the sulfur-containing amino acids methionine or cystine, decreases the incidence of massive hepatic necrosis and permits the rat to survive for a long period of time. These rats then acquire the sterility and chronic muscular dystrophy typical of vitamin E deprivation, but at death there are no lesions in the liver.

4. The addition of adequate amounts of α-tocopherol invariably prevents massive hepatic necrosis, enabling the rat to survive for a long period of time and to reproduce under conditions that are associated with fatty deposition in the liver and diffuse hepatic fibrosis.

5. Confirmatory evidence is given that methionine prevents, and cystine intensifies, fatty infiltration of the liver and diffuse hepatic fibrosis.

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THE RESPONSE OF COBALT-DEFICIENT LAMBS TO LIVER EXTRACT AND VITAMIN B_{12}

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Becker and Smith ('51) presented evidence that an anti-pernicious anemia liver extract gave a positive curative effect in cobalt-deficient lambs when injected, but not when fed at the same level. The curative factor in the liver extract could not have been cobalt per se. This paper summarizes further work done toward identifying the effective factor in the liver extract.

METHODS

The 25 lambs used in this study were selected from a carload lot of whiteface western feeders and averaged 48 lb. at the start. The general methods of management and housing have been described previously (Smith et al., '50). After parasite treatments the animals were fed a low-cobalt ration of shelled yellow corn, powdered milk, and especially selected grass hay of low cobalt content. The lambs were gradually brought on feed until they were consuming an average of 450 gm of corn, 540 gm of hay, and 90 gm of milk powder per day, a ration that meets the Morrison feeding standards for growing lambs. On this level of feed intake they reached an average weight of 78 lb. before deficiency symptoms became evident. They were continued on these feeds until signs of cobalt deficiency — anorexia, loss in weight, and anemia — were well established (approximately 6 months). At this time they were divided into treatment groups as indicated later.

From this time on each lamb was offered a daily ration of 300 gm of corn, 60 gm of milk powder, and 400 gm of hay. As the deficiency progressed, less and less of this ration was consumed. This was especially true of the concentrate intake. The corn used in this study averaged about 0.01 p.p.m., the milk powder 0.01, or less, p.p.m., and the hay ranged from 0.05 to 0.07 p.p.m. of cobalt on a dry basis. This particular hay averaged a little higher in cobalt than that previously used. Plain salt and tap water were always available. The lambs were kept in individual pens bedded with wood shavings. During treatment periods body weights and hemoglobin determinations were made weekly. Daily records were kept of feed intakes.

All lambs were not started on the indicated treatments at the same time. Rather, a group of comparable deficient lambs were assigned to selected treatments, the outcome of which determined the next set of treatments. The order of these treatments is indicated under the discussion of results.

The liver extract and the various fractions used in this experiment all came from a one-liter sample of a commercial preparation [1] stated to contain 15 U.S.P. units of antipernicious anemia activity per milliliter. The vitamin B_{12} activity of the original extract, as well as of all fractions, was determined microbiologically using the procedure described by Peeler et al. ('49). According to this assay, the original liver extract contained approximately 20 μg of B_{12} activity per milliliter. A sample of this extract was separated into 24 fractions using the counter-current distribution technic originally described by Craig ('44) and modified by Combs et al. ('50). This fractionation technic was slightly modified in that 32.5% phenol and 2% acetic acid were used and the final extraction was made with carbon tetrachloride. A further liver extract sample was treated so as to destroy its B_{12} activity. This was done by raising the sample to pH 12.0 with 20% KOH and autoclaving for 30 minutes at 15 lb. pressure. The pH was returned to 6.0 using 5% HCl. An assay showed total

[1] Courtesy of the Eli Lilly Company.

destruction of B_{12} activity. This sample was halved and an amount of crystalline B_{12} equal to that assayed in the same volume of original extract was added to one-half, thus restoring the B_{12} activity.

In all cases the treatment period was of two weeks' duration. All compounds tested were injected subcutaneously twice each week. The liver extract, "liver extract minus B_{12}," "liver extract minus B_{12} plus B_{12}," and "sham-treated liver extract" were injected at the rate of 1 ml of original liver extract per day. Each of the 24 fractions was evaporated by low temperature under reduced pressure to a volume of 84 ml and each fraction tested was divided equally among three lambs (necessitating an injection rate of 2 ml per day). The vitamin B_{12} [2] was dissolved in water to give a concentration of 20 μg per milliliter and injected to give the indicated total dosage. Cobalt as the chloride was given orally twice weekly at the rate of 1 mg per day.

RESULTS

In determining a positive or negative response to a particular treatment, we have placed most reliance on the appetite and weight responses rather than on hemoglobin response. The appetite and weight responses are more sensitive in that they quickly follow by a matter of days a favorable treatment, and the magnitude of these responses, particularly of appetite, is large. Hemoglobin responses in cobalt-deficient lambs fed adequate cobalt salts are generally upward but slow, frequently lagging behind the appetite and weight responses by some weeks, and are often erratic. This was noted by Filmer ('33) and Filmer and Underwood ('37) several years ago, and more recently by Ray et al. ('48). Our own observations bear this out.

The results of this study are summarized in tables 1, 2, and 3. Table 1 presents the initial results, involving chiefly a confirmation of the presence of a curative factor for cobalt-

[2] Merck's Cobione.

deficient lambs in liver extract and identifying those fractions of the extract in which the curative factor was concentrated. Table 2 contains those data accumulated from the latter part of the study designed to identify the effective factor that was concentrated in some of the liver extract fractions. Many but not all of the animals appearing in table 2 had received

TABLE 1

Appetite and weight responses of cobalt-deficient lambs when injected with liver extract and its various fractions

TREATMENT	NO. OF LAMBS	AVE. CONCENTRATE INTAKE		AVE. BODY WEIGHT		TOTAL VITAMIN B_{12} INJECTED PER LAMB
		Pre-treatment week	5th week	Pre-treatment week	5th week	
		gm/day		*lb.*		*μg*
Cobalt fed	4	109	346	53	60	...
Liver extract	6	61	345	55	61	275
Liver extract (sham treatment)[1]	3	52	252	54	60	200
Liver extract						
Fraction 0	1	47	61	52	49	15
Fraction 3	1	86	20	58	51	15
Fraction 6	1	139	96	52	46	25
Fraction 9	2	88	107	57	55	75
Fraction 12	2	82	185	58	59	150
Fraction 15	3	57	246	56	60	175
Fraction 18	2	84	224	68	71	140
Fraction 21	2	72	82	60	56	30
Fraction 24	2	64	..[2]	59	..[2]	2

[1] Extract given all treatments as in fractionation except the actual fractionation.
[2] Both lambs died during treatment period.

some previous treatment. Those that had responded positively to a previous treatment were again reduced to a cobalt-deficient state prior to the second treatment. Some had not responded at all to a previous treatment and some had received no previous treatment. Each treatment group indicated in table 2 was balanced in so far as possible with animals from the above three categories.

Table 1 gives treatment-group averages contrasting the concentrate (corn and milk powder) intakes and body weights of the week prior to treatment with those of the 5th week following the initial treatment. The 5th week was chosen, since this, for the most part, represented the week of maximum response in weight gains. It will be noted that those lambs fed cobalt salts, representing a positive control group, increased significantly in appetite and body weight, as expected, as did those that received injections of the original liver extract. Before starting the treatments with liver frac-

TABLE 2

Appetite and weight responses of cobalt-deficient lambs injected with vitamin B_{12}

TREATMENT	NO. OF LAMBS	AVE. CONCENTRATE INTAKE		AVE. BODY WEIGHT		TOTAL VITAMIN B_{12} INJECTED PER LAMB
		Pre-treatment week	5th week	Pre-treatment week	5th week	
		gm/day		*lb.*		*μg*
Liver extract minus B_{12} [1]	5	72	25 [2]	59	56	0
Liver extract minus B_{12} plus crystalline B_{12}	6	59	294	58	64	255
Crystalline B_{12}	6	52	254	59	64	300

[1] B_{12} activity destroyed by autoclaving in an alkaline medium.
[2] One lamb died during treatment period.

tions obtained in the counter-current distribution, it was thought wise to determine if the chemical treatments used in the fractionation destroyed the active factor. Thus a liver extract sample (sham treatment) was subjected to all chemical treatments used in the counter-current distribution except the actual fractionation. This "sham-treated" liver extract, when injected into cobalt-deficient lambs, resulted in appetite and weight increases, showing that the active factor was not destroyed. Since facilities did not permit the assay of all 24 fractions of the liver extract, it was decided to test every third fraction with one lamb each; then more animals would be tested with those fractions that gave a positive response.

This was in keeping with the objective of the study, which was to concentrate the active principle of the liver extract, prior to actual identification. The results given in table 1 show that the active principle was concentrated in fractions 12 to 18 inclusive. The anmals tested were too few to permit the conclusion that one of these three fractions is any better than the others. However, there is a suggestion that fractions 15 and 18 were superior to 12.

Table 1 also summarizes the vitamin B_{12} activity of the various liver fractions tested, and the correlation between the vitamin B_{12} activity of the fractions and the lambs' responses is obvious. This correlation strongly indicated that the active principle of the liver extract was vitamin B_{12}. This observation was at variance with our previous limited observation (Becker et al., '49) that cobalt-deficient lambs did not respond to crystalline vitamin B_{12}. The amounts of B_{12} previously injected were lower than 150 μg which, as table 1 indicates, is approximately the minimum curative dose. Thus the next series of studies were designed to test the prophylactic effects of higher levels of vitamin B_{12}.

Seventeen lambs were divided, as equally as possible, into three treatment groups. One group received the liver extract in which the vitamin B_{12} activity had been chemically destroyed. Another received a sample of this treated extract to which the vitamin B_{12} activity had been restored to original levels by the addition of crystalline B_{12}, and the third group received crystalline B_{12} alone.

Those lambs injected with liver extract in which the B_{12} activity had been destroyed decreased in concentrate intake and body weight. When the B_{12} activity of the treated liver extract was restored, the lambs responded in appetite and weight gains, as did those that received crystalline B_{12} alone.

The average responses to the various treatments with respect to hemoglobin levels of the blood are summarized in table 3. In general, those treatments that resulted in appetite and weight gains also resulted in increased hemoglobin

concentrations, although these increases were not large by the 5th week and were still below normal levels, even in those lambs fed cobalt salts (Becker and Smith, '50).

These results show that cobalt-deficient lambs will respond favorably in appetite, weight gains, and hemoglobin levels, if they are injected with sufficiently large amounts of vitamin

TABLE 3

The hemoglobin levels of cobalt-deficient lambs given various treatments of liver extract and vitamin B_{12}

TREATMENT	NO. OF LAMBS	AVE. HEMOGLOBIN LEVEL	
		Pretreatment week	5th week
		gm/100 ml blood	
Liver extract	6	7.2	8.2
Liver extract (sham-treated)	3	8.0	9.5
Liver extract			
Fraction 0	1	7.3	6.9
Fraction 3	1	6.2	5.6
Fraction 6	1	7.6	6.5
Fraction 9	2	6.7	6.3
Fraction 12	2	8.1	7.9
Fraction 15	3	7.1	8.4
Fraction 18	3	7.3	7.8
Fraction 21	2	9.5	6.9
Fraction 24	2	5.8	[1]
Liver extract, minus B_{12}	5	8.4	8.5
Liver extract, minus $B_{12} + B_{12}$	6	6.5	8.5
Crystalline B_{12}	6	8.9	10.1
Cobalt	4	5.8	8.4

[1] Both lambs died.

B_{12}. Although this study was not designed to determine the minimum curative dose of B_{12}, the results shown in table 1 suggest that it is approximately 150 μg under the conditions of this study. In terms of the minimum levels of vitamin B_{12} required to obtain a favorable response in pernicious anemia in man, this is a relatively large amount.

Whether the effectiveness of the liver extract resides solely in its B_{12} activity cannot be ascertained from these data. The comparable responses of lambs injected with "liver extract minus B_{12} plus B_{12}" and with crystalline B_{12} only (table 2)

suggest that most, if not all, of the activity of the liver extract was due to its B_{12} content.

DISCUSSION

In the light of these more extensive observations, it seems certain that our previous failure (Becker et al., '49) to obtain a response in cobalt-deficient lambs with vitamin B_{12} injections was due to the use of inadequate amounts of the vitamin. This was unavoidable, since the supplies of vitamin B_{12} were then extremely limited and we had available a total of only 200 μg.

Hale et al. ('50) reported that two cobalt-deficient lambs responded favorably to a complete B-vitamin supplement but that injections of vitamin B_{12} gave an unsatisfactory response. A total of 5 cobalt-deficient lambs were injected with B_{12}. Four of these lambs received a total of 25, 30, 40, and 40 μg each, respectively, and in view of the data of the present paper these amounts were inadequate to give a favorable response. The 5th lamb was injected with 100 μg of B_{12} per day for 4 weeks. During the 4-week period the hemoglobin level rose from 6.57 to 8.09 gm/100 ml of blood and the lamb gained 5 lb. Hale et al. interpreted this as an unsatisfactory response. We believe that the opposite interpretation could as well be made. Either interpretation would, of course, be more valid if supported by larger numbers of observations. Keener and Percival ('50) reported a negative response in two cobalt-deficient lambs injected with a commercial preparation of liver extract; whether these very limited observations are in discord with our findings cannot be ascertained, inasmuch as the vitamin B_{12} activity of the liver extract is unknown. That the antipernicious anemia liver extracts on the market vary widely in vitamin B_{12} activity has been shown by Peeler et al. ('49), who reported a range in 10 extracts of from 87 to 2,170 mμg per U.S.P. unit.

The presently available data support the seemingly obvious theory that dietary cobalt has for at least one of its functions

the production of vitamin B_{12} in the rumen of sheep, probably by microbial synthesis, and that cobalt deficiency is at least partially a vitamin B_{12} deficiency. The higher vitamin B_{12} activity of the rumen contents of cobalt-fed sheep, as compared to that of cobalt-deficient sheep, has been reported by Hale et al. ('50) and is in keeping with this theory. At the moment the limited data of Hale et al. indicating the positive response of cobalt-deficient lambs to a complete B-vitamin supplement, excepting B_{12}, is difficult to explain. Hale suggested that perhaps the organisms that fail to grow due to a lack of cobalt are concerned with B-vitamin synthesis. Another possibility is a sparing effect of some B vitamin on vitamin B_{12} supplies.

SUMMARY

The curative activity of an antipernicious anemia liver extract when injected into cobalt-deficient lambs has been confirmed. Separation of this liver extract into 24 fractions by the counter-current distribution technic concentrated the active principle in fractions 12 to 18 inclusive. There was a high correlation between the vitamin B_{12} activity of these liver extract fractions and the appetite, weight, and hemoglobin responses in cobalt-deficient lambs, which led to a re-evaluation of the curative effects of pure vitamin B_{12}. When large enough doses, 150 μg or more, of B_{12} were injected, the cobalt-deficient lambs responded favorably in appetite, body weight, and hemoglobin levels.

The presently available data show that vitamin B_{12} is an important intermediary in cobalt metabolism in sheep.

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SEX DIFFERENCES IN THE GROWTH OF YOUNG RATS AND SURVIVAL OF ADULT RATS FED PROTEIN-DEFICIENT DIETS

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As far as the present authors are aware, the only investigation heretofore published on the subject of differential sex response to diets inadequate in quantity or quality of essential nutrients was that reported by Hartwell ('25). This author, working with rats, noted that females grew better than males on diets deficient in protein or in vitamin B_1. Later, several observations have been incidentally mentioned in connection with other studies to the effect that females withstand the effects of dietary deficiencies better than males. In a study on endochondral ossification Levy and Silberberg ('46), working with mice, observed that the males were more sensitive than the females both to pantothenic acid and riboflavin deficiencies. Carlson and Hoelzel ('48), in studies with rats on certain inadequate rations, observed that the period between weaning and sexual maturity was the most difficult for the offspring of the vegetarians to bridge. About one-half of the males died during this period. The females fared better. In a study on the effect of the fat level in the diet on general nutrition, Sheer, Codie and Deuel ('47) noted weight loss, mortality, and recovery in young adult rats maintained on a diet severely restricted in calories. They observed that after an experimental period of 12 weeks the males had lost from 40 to 50% of their initial weight, while the females had lost

only 30 to 40%. Keys ('46) has pointed out that during the 1945 famine in Western Holland the famine mortality was considerably higher among males than females, and that there are indications of similar findings in the siege of Leningrad (1942–1944), and in the Indian famines. Valoras ('46) reported that during the war famine in Athens and Piraeus, Greece, 1941–1943, adult women survived starvation better than did adult men.

EXPERIMENTAL

Sex differences in growth

In connection with some comparatively recent feeding experiments with young rats to determine the growth-promoting values of certain foods it was observed that the females showed greater weight gains than the males when fed diets supplying protein either of generally recognized low nutritional value, or adequate in quality but at suboptimum levels in the diet. On the other hand, when the diets supplied protein adequate in quality and quantity, together with the non-protein essential dietary factors, the males consistently surpassed the females in weight gains.

These observations led to an examination of the records and results obtained in this laboratory over a period of years in connection with feeding experiments, involving the use of approximately 1,000 rats, to evaluate the protein quality of a wide range of foods at different protein levels in the diet. With few exceptions, each feeding experiment extended through 6 weeks and, in most cases, included 12 young rats equally divided with respect to sex and ranging in weight at the start from 40 to 60 gm. In several instances as many as 24 animals were used in one experiment. The diets were adequate with respect to the non-protein dietary factors known at the time to be essential.

Table 1 shows the weight gains of young male and female rats in a representative series of experiments in which the diets furnished protein, at various percentage levels, derived

from foods generally recognized as sources of protein of high nutritional value. It will be noted that the only instances in which males did not gain more weight than the females were those when the diets supplied either protein of good quality but at a very low level (e.g., dried egg at the 4.5% level, and casein at the three lower levels), or foods the protein of which had been impaired by commercial processing; namely, peanut and cottonseed meals. The data on dried whole egg, which contains protein of excellent quality, serve as remarkable illustrations of sex differentiation at different protein levels in the diet. With decreasing protein levels, ranging from 25% to 7.5%, the ratios between the weight gains of the males and those of the females decreased successively in the following order: 1.62, 1.58, 1.36, and 1.14. However, at the low level of 4.5% protein the females showed greater gains than the males, the ratio being 0.87%. It is of interest to note the better growth rate at intermediate levels on most "good quality" proteins.

Also of interest are the lower weight gains obtained, both for the males and the females, with the commercial samples of cottonseed and peanut meals as compared with the gains of the animals fed the cottonseed meal prepared by the flotation process (Vix, Spadero, Westbrook, Crovetto, Pollard and Gastrock, '47),[1] and the samples of peanut meal prepared in our laboratory from raw, Virginia peanuts. Not only were the weight gains of both sexes lower when the animals were provided with the commercial meals, but the order of the gains with reference to sex was reversed. With the cottonseed meal prepared by the flotation process, the average weight gains of the males were definitely greater than those of the females, whereas with the commercial meals the females did better than the males. Similarly, in the case of the peanut meals the males fared better than the females on the meals prepared in the laboratory but with the commercial meals,

[1] The cottonseed meals prepared by the flotation process were kindly supplied by the Southern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, United States Department of Agriculture.

TABLE 1

Sex differences in the growth of young rats fed diets supplying protein of good quality at various percentage levels

MATERIAL	PROTEIN IN DIET	WEIGHT GAINS IN 6 WEEKS	
		Males	Females
	%	*gm*	*gm*
Dried whole eggs	28	157	106
Dried whole eggs	25	168	104
Dried whole eggs	20	171	108
Dried whole eggs	15	211	139
Dried whole eggs	15	177	119
Dried whole eggs	10	183	134
Dried whole eggs	10	216	143
Dried whole eggs	7.5	126	110
Dried whole eggs	7.5	136	72
Dried whole eggs	4.5	60	69
Casein (Labco)	40	181	130
Casein (Labco)	35	174	107
Casein (Labco)	28	160	126
Casein (Labco)	25	161	125
Casein (Labco)	17.5	194	155
Casein (Labco)	15	202	144
Casein (Labco)	10	115	124
Casein (Labco)	9	99	101
Casein (Labco)	4.5	42	42
Cottonseed meal (flotation process)	28	122	93
Cottonseed meal (flotation process)	25	158	99
Cottonseed meal (flotation process)	20	142	106
Cottonseed meal (flotation process)	15	150	75
Cottonseed meal (flotation process)	10	144	105
Cottonseed meal (flotation process)	10	162	107
Cottonseed meal (flotation process)	9.6	139	120
Cottonseed meal (flotation process)	8	116	102
Cottonseed meal (commercial)	10	75	78
Cottonseed meal (commercial)	9	83	88
Dried skim milk	28	166	115
Dried skim milk	25	179	95
Dried skim milk	20	146	130
Dried skim milk	15	133	120
Dried skim milk	15	140	106
Dried skim milk	15	181	118
Dried skim milk	15	192	130
Dried skim milk	12	163	136
Dried skim milk	10	135	106
Dried skim milk	10	128	106
Dried skim milk	9	161	127
Wheat germ (defatted)	28	132	111
Wheat germ (defatted)	25	149	96
Wheat germ (defatted)	20	173	103
Wheat germ (defatted)	17	163	100
Wheat germ (defatted)	15	172	117
Wheat germ (defatted)	10	152	112
Corn germ (defatted)	15	136	92
Corn germ (defatted)	10	103	99
Corn germ (defatted)	10	106	97
Corn germ (defatted)	10	112	91
Corn germ (defatted)	10	113	89
Peanut meal (commercial)	10	80	90
Peanut meal (commercial)	9	70	85
Peanut meal (raw, prepared in the laboratory)	10	126	112
Peanut meal (autoclaved, prepared in the laboratory)	10	126	108
Soybean meal (commercial)	28	129	85
Soybean meal (commercial)	25	122	94
Soybean meal (commercial)	20	133	80
Soybean meal (commercial)	15	120	70
Lean pork	10	121	114

which had lower nutritive values, the females made greater gains. The lower values found for the commercial meals are of particular interest in that they reflect the injurious effect of heat and possibly other factors to which the materials had been subjected during the process of their production. Cottonseed meal prepared by the flotation process is not subjected to high temperature, and the raw peanut meal was prepared in our laboratory by ether extraction of ground, raw, Virginia peanuts. Incidentally, it is of interest to note that autoclaving the peanut meal at 15 pounds pressure for 30 minutes had no appreciable effect on the nutritive value of its protein. In this respect, raw peanut meal differs from raw soybean meal, inasmuch as the latter is improved by moderate heating.

In table 2 are given the weight gains of young male and female rats supplied chiefly with rations either of low or medium protein nutritional value, or containing protein at suboptimum levels. The weight gains obtained with these protein-deficient diets are in striking contrast to those given in table 1, not only with reference to the total weight gains of both sexes but to the gains of the females, which surpassed those of the males. Of particular interest in this respect is the marked reversal in the order of sex differentiation in growth, and the low weight gains of both sexes when the animals were supplied with the several different raw soybean preparations as compared with the much higher values obtained with the commercial, heat-processed soybean meals (table 1). It is generally recognized that raw soybean meal is an unsatisfactory source of dietary protein, and that it requires adequate heat processing to develop its maximum protein nutritional value.

At the 4.5% protein level in the cereal grain diets the females showed greater weight gains than the males except for rye, which resulted in practically the same gains for both sexes. At the higher levels, however, the gains of the males exceeded those of the females, except for the results obtained with corn and barley. With rolled oats at the 9.5 and 12% protein levels the gains of the males were definitely greater

than those of the females. Rolled oats has been shown to have a higher protein biological value than other cereals (Jones, Caldwell and Widness, '48).

TABLE 2

Sex differences in the growth of young rats fed diets varying in the quality and quantity of the protein

MATERIAL	PROTEIN IN DIET	WEIGHT GAINS IN 6 WEEKS	
		Males	Females
	%	gm	gm
Soybean meal, raw	18	68	84
Soybean meal, raw, alcohol extracted	10	21	40
Soybean meal, raw, ether extracted	10	28	35
Soybean meal, raw, isolated protein	18	25	32
Corn, yellow	8.1	45	54
Corn, yellow	7.5	39	50
Corn, yellow	5.0	20	24
Corn, yellow	4.5	12	22
Corn, yellow	4.5	5	7.5
Barley, pearled	7.5	39	48
Barley, pearled	4.5	18	21
Wheat, hard, winter	7.5	45	41
Wheat, hard, winter	4.5	14	18
Wheat, soft, spring	6.0	29	33
Rye	7.5	64	61
Rye	6.0	44	39
Rye	4.5	34	35
Oats, rolled	12	111	101
Oats, rolled	9.5	105	98
Oats, rolled	4.5	33	38
Corn gluten	18	84	71
Corn gluten	10	53	46
Corn gluten	8	35	32
Corn gluten	6	4	13
Wheat flour, patent	9	17	21
Wheat flour, patent, 85 parts + soybean flour 15 parts	9	98	88
Wheat flour, patent, 85 parts + dried skim milk 15 parts	9	89	81
Wheat flour, patent, 90 parts + casein 10 parts	9	156	131
Wheat flour, patent, 85 parts + casein 15 parts	9	161	136

The relation between sex differentiation and protein quality is also reflected in the results of the supplementation experiments with wheat flour. With wheat flour alone the females

showed somewhat greater gains than the males, but when the flour was supplemented with soybean flour, skim milk powder, or casein, the males showed definitely greater weight gains.

In table 3 is shown a reversal in the order of sex differentiation in the growth of the same young animals when they were supplied consecutively over two periods of 42 days each, first, with a corn diet deficient in the quality and quantity of its protein, and then with a high protein casein diet. Twelve young rats, equally divided with respect to sex and ranging in weight from 53 to 60 gm, were fed during the first period

TABLE 3

Lot 249 — Sex differences in the growth of the same lot of young rats fed over two consecutive periods a deficient protein corn diet and a high protein casein diet (duration of each period — 42 days)

SEX	CORN DIET PERIOD (5% protein level)			CASEIN DIET PERIOD (12% protein level)		
	Average original weight	Average final weight	Average gain in 42 days	Average starting weight	Average final weight	Average gain in 42 days
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Males	59	71	12	71	185	114
Females	56	72	16	72	167	95

with the same deficient diet as is described later (p. 472). for lots 247 and 248, except that 55 parts of the ground corn were fed to supply protein at the low level of 5% in the diet.

During the first period when the low-level protein-deficient diet was used all of the females exceeded the males with respect to weight gains. With the high-level casein diet during the second period, however, there was a definite reversal in the comparative rate of growth of the sexes, and by the end of this period the weight gains of the males had definitely exceeded those of the females.

Sex differences in survival

The foregoing observations on sex differentiation in growth were confined to the effects of different diets on the rate of growth of young rats. The following experiments were con-

ducted to study the comparative survival of adult male and female rats and their resistance to the effect of subsisting on a diet deficient in the quantity and quality of the protein.

The survival experiments were conducted with two lots of adult albino rats equally divided as to sex. Lot 247 consisted of 12 animals. At the start the males ranged in weight from 408 to 518 gm, and averaged 453 gm. The females varied from 245 to 290 gm, and averaged 268 gm. The animals were housed in individual cages and provided ad libitum with distilled water and a diet deficient with respect to the quantity and quality of the protein. The diet was satisfactory with respect to known dietary factors other than protein. The protein was supplied by a poor quality of ground yellow corn which was incorporated in the diet to provide protein at a 4% level. The diet had the following percentage composition: Yellow corn 44, dextrin 44, inorganic salt mixture 2 (Hubbell, Mendel and Wakeman, '37), cod liver oil 2, and corn oil 8. A vitamin solution was incorporated in the diet to provide the following constituents in milligrams per 100 gm of diet: thiamine hydrochloride, 0.2; niacin, 1; pyridoxine hydrochloride, 0.2; riboflavin, 0.3; calcium pantothenate, 0.3; and choline hydrochloride, 10. These substances, in aqueous solution, were added to the dextrinized starch and the mixture was dried at 50°C. to the original weight of the starch.

The animals fed the deficient diet lost weight promptly. Gradually they began to show other evidences of malnutrition — yellowing and roughening of the hair, emaciation, hemorrhagic condition of the eyes and general weakness.

At the end of the experimental period, which lasted 211 days, only one of the original 6 males was alive, and it could have survived only a few days longer. Three had died and two were killed because they were in a moribund condition. The average length of survival of the male rats was 149 days, and their loss of weight ranged from 36 to 60% and averaged 51%. On the other hand, three of the 6 females were alive at the end of the period, two of which were in fairly good condition and had lost an average of only 13% of their original

weight. The other, weak and emaciated, had lost 50% of its original weight and could have lived only for a short time. The 4th animal died suddenly of a hemmorrhage on the 183rd day of the experiment, and the 5th, in a moribund condition, was killed on the 143rd day, after having lost 50% of its weight. The 6th rat died on the 197th day in a weak and emaciated condition. The average length of survival of the female rats was 231 days, and their loss of weight ranged from 25 to 50% with an average of 33%.

A second experiment on survival (lot 248) was conducted with 11 males and 10 females. At the start the males ranged in weight from 230 to 416 gm and averaged 354 gm. The females ranged from 198 to 359 gm and averaged 268 gm. The animals were given the same deficient corn diet as was used for lot 247. They promptly declined in weight and soon manifested a yellowing and roughening of the hair. Later, a hemmorrhagic condition of the eyes developed which in some cases resulted in blindness. Affliction of the eyes generally developed within three to 4 months after they had been placed on the deficient diet. Sores on the feet and tails were common. Diarrhea was quite prevalent. The average length of survival was 161 days for the females and 139 days for the males, after weight losses of 44 and 52%, respectively.

SUMMARY AND CONCLUSIONS

A review of the results of a large number of feeding experiments conducted in this laboratory over a period of several years, involving somewhat over 1,000 young rats approximately equally divided as to sex and ranging in weight at the start from 50 to 60 gm, has revealed that almost invariably the females showed greater weight gains than the males when the diets were inadequate with respect to either the quality or quantity of the protein, but known to be otherwise satisfactory. On the other hand, in similarly conducted experiments in which the animals were provided with diets adequate in all respects, the males consistently surpassed the females in weight gains.

Foods supplying protein of high nutritional value included dried whole eggs, casein, cottonseed meal (flotation process), dried skim milk, wheat germ, corn germ, peanut and soybean meals, and lean pork. Protein material of low to medium nutritional values included a number of raw soybean preparations, several cereal grains, patent wheat flour, and commercial corn gluten. The materials were incorporated in the diets to supply protein ranging from 4.5 to 28%.

When provided with protein of good nutritional quality the rate of growth for both sexes was greater at intermediate levels (below 20%) than at higher levels. At the higher protein levels the males surpassed the females but at the low level of 4.5% the females invariably equalled or surpassed the males in weight gains.

When diets were used supplying protein ranging from low to medium in nutritive value, such as the several preparations of raw soybean meal and patent wheat flour, at 9% protein in the diet, the females fared better than the males. On the other hand, when the diet was enriched with 10 to 15 parts of soybean flour, dried skim milk, or casein, and the mixtures fed at the same protein level (9%), the males exceeded the females in weight gains.

With decreasing levels of high quality protein (dried whole egg) in the diet, ranging from 25 to 7.5%, the ratios between the weight gains of the males and those of the females decreased successively in the following order: 1.62, 1.58, 1.36, and 1.14, until at the low level of 4.5% protein the females showed greater gains than the males in the ratio of 1 to 0.87 gm, respectively. Similar decreasing ratios between the sexes were observed when casein supplied the protein in the diet.

The number of animals used in the survival experiments was rather small to provide a basis for generalization. However, the results definitely indicate that adult female rats have a greater ability to survive than males when restricted to a diet grossly inadequate with respect to the quality and quantity of its protein.

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URINARY EXCRETION OF ESSENTIAL AMINO ACIDS BY HUMAN SUBJECTS FED DIETS CONTAINING PROTEINS OF DIFFERENT BIOLOGICAL VALUE [1]

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Relatively little information is available regarding the urinary excretion of amino acids by man as related to the quality of ingested protein. Steele et al. ('47) determined the excretion of 16 amino acids in the urine of 4 college women who ingested diets low in nitrogen, containing either soybeans or eggs as the source of protein. They reported that the amount of any amino acid excreted was unchanged by alternate ingestion of these two proteins. Several samples of urine lacked one or more amino acids and leucine was found only when the subjects were in negative nitrogen balance.

Harvey and Horwitt ('49) determined the excretion of the essential amino acids by 20 men who subsisted on a closely regulated institutional diet for three years. The intake of protein was 55 gm per day, approximately half of which was provided as meat. All of the essential amino acids were found in the urine. Excretions of "free" amino acids (microbiologically available before hydrolysis) ranged from 0.9 mg/day for isoleucine to 38.0 mg/day for histidine. Woodson et al. ('48) investigated the urinary excretion of 15 amino acids by human subjects. They reported the excretion of all

[1] This investigation was supported in part by a grant from the Office of Naval Research, Contract NR111-192.

of the essential amino acids in the free form and that histidine excretion was much greater than any other.

Recently in this laboratory, 6 proteins were evaluated for human nutrition (Hawley et al., '48). It was decided to utilize this opportunity to make some observations on the urinary excretion of the "free" essential amino acids. The proteins spanned nearly the whole scale of biological value and it was hoped that some correlation might be established between this value and the wastage of the essential amino acids in the urine.

METHODS

The subjects were men and women students 21 to 30 years of age. Five subjects of each sex participated in the first two dietary periods; in the remaining 4 periods one of the women was absent. The dietary periods varied in length from 4 to 7 days; the urines were collected on the last two days, diluted to a volume of 2,000 ml and stored in the cold under toluene until analyzed. Aliquots from each day were combined for the amino acid determinations in the urine of each male subject. Because of lack of time and facilities, all urines from the females were pooled and analyzed as a single sample.

The order of the 6 dietary periods and the average total nitrogen intake per day were: Non-protein, 0.29 gm N; casein, 4.88 gm N; non-protein, 0.23 gm N; fresh egg, 4.54 gm N; wheat gluten, 5.33 gm N; dried beef, 4.96 gm N. The nitrogen contributed by the test protein ranged from 92% for glutein to 96% for beef. The remainder of the nitrogen intake was derived chiefly from applesauce, butter and the components of salads. Calorie intake was adjusted for each individual so that body weight remained approximately constant. The protein intake was designed to furnish 5% of total calories. Details of the investigation of the biological values of these test proteins are described elsewhere (Hawley et al., '48).

The "free" essential amino acids were determined microbiologically, using the media given in table 1. The medium labeled "General purpose" was used for the following organisms: *Streptococcus faecalis* (9790), *Streptococcus faecalis R* (8043), *Lactobacillus delbruekii LD5* (9595), *Lactobacillus casei E* (7469), and *Lactobacillus arabinosus 17-5* (8014). The modifications used for *Leuconostoc mesenteroides P-60* (8042) and *Lactobacillus fermenti 36* (9338) are indicated in table 1. All organisms were obtained from the American Type Culture Collection.

The average recovery of the 10 essential amino acids [2] added to urine was 105% (range, 93% to 124%). Previous decomposition of urea by urease, with subsequent removal of ammonia, and decarboxylation of urinary amino acids by ninhydrin, failed to alter the percentage recovery of added amino acids. Histidine, leucine, methionine, phenylalanine, tryptophan and valine were determined with two species or organisms each; arginine, isoleucine, lysine and threonine were each determined with a single species.

RESULTS

The daily excretion of essential amino acids is summarized in table 2. In most experiments the males excreted larger amounts than the females but on the basis of body weight (δ ave. = 73.2 kg; φ ave. = 64.6 kg) or metabolic body size (δ ave. = 25.3 $kg^{3/4}$; φ ave. = 22.8 $kg^{3/4}$) no sex difference is evident.

The excretion of each amino acid by the males, expressed as a percentage of the amount ingested, is given in table 3. The average ingestion of each amino acid was derived from the average intake of each protein and its amino acid composition as published by the Bureau of Biological Research of Rutgers University ('50). In order to save space, similar data for the female subjects were omitted from the table.

[2] The amino acids were supplied by Merck and Co., Rahway, N. J.

TABLE 1

Composition of media used in microbiological determination of urinary amino acids
(Amounts in 1,000 ml final assay medium)

		GENERAL PURPOSE	LEUCONOSTOC MESENTEROIDES P-60	LACTOBACILLUS FERMENTI 36
Adenine $SO_4 \cdot 2H_2O$	mg	10	10	10
Guanine $HCl \cdot 2H_2O$	mg	10	10	10
Uracil	mg	10	10	10
Xanthine	mg	10	10	10
Glucose	gm	20	20	20
Na acetate	gm	12	12	12
NH_4Cl	gm	3	3	3
Na citrate	gm	25	25	25
KH_2PO_4	mg	500	500	500
K_2HPO_4	mg	500	500	500
$MgSO_4 \cdot 7H_2O$	mg	200	200	200
$FeSO_4 \cdot 7H_2O$	mg	10	10	10
$MnSO_4 \cdot 4H_2O$	mg	10	10	10
NaCl	mg	10	10	10
p-Aminobenzoic acid	μg	100	100	100
Biotin	μg	5	5	5
Choline chloride	μg	2,000	2,000	2,000
Folic acid	μg	10	10	10
Inositol	μg	2,000	2,000	2,000
Nicotinic acid	μg	1,000	1,000	1,000
DL-Ca-pantothenate	μg	1,000	1,000	1,000
Pyridoxine·HCl	μg	1,000	1,000	1,000
Pyridoxamine·2HCl	μg	400	400	400
Riboflavin	μg	1,000	1,000	1,000
Thiamine chloride	μg	500	500	500
DL-Alanine	mg	300	1,200	480
L-Arginine·HCl	mg	200	200	200
DL-Aspartic acid	mg	200	200	200
L-Asparagine	mg	100	100	100
L-Cystine	mg	200	200	200
L-Glutamic acid	mg	400	400	400
Glycine	mg	200	200	400
L-Histidine·HCl·H_2O	mg	200	200	200
L-Hydroxy proline	mg	200	200	200
DL-Isoleucine	mg	200	200	400
DL-Leucine	mg	200	200	300
L-Lysine·HCl·H_2O	mg	200	200	200
DL-Methionine	mg	200	200	200
DL-Norleucine	mg	200	200	200
DL-Phenylalanine	mg	200	200	200
L-Proline	mg	200	200	200
DL-Serine	mg	200	200	200
DL-Threonine	mg	200	400	300
DL-Tryptophan	mg	400	300	300
L-Tyrosine	mg	200	200	200
DL-Valine	mg	200	200	400

TABLE 2

Average urinary "free" amino acid excretions on various diets
(Milligrams per day)

AMINO ACIDS	SEX	DIETS				
		Non-protein	Gluten	Casein	Beef	Fresh egg
Arginine	♂	12.8	10.8	18.6	15.8	13.8	16.0
	♀	8.2	7.6	14.2	14.8	8.6	12.4
Histidine	♂	..	80	118	68	70	60
	♀	..	84	140	70	66	56
Isoleucine	♂	1.2	1.4	2.2	1.8	2.2	3.0
	♀	1.0	1.0	1.8	1.8	2.0	2.4
Leucine	♂	6.2	6.4	7.8	7.4	10.8	8.2
	♀	5.4	5.2	5.4	7.2	7.4	5.6
Lysine	♂	7.2	6.8	6.6	10.6	11.4	7.2
	♀	5.6	7.8	5.4	14.2	10.2	6.0
Methionine	♂	0.56	0.44	0.60	0.92	0.54	0.62
	♀	0.48	0.36	0.02	0.86	0.88	0.54
Phenylalanine	♂	7.0	8.0	10.6	9.2	9.6	11.2
	♀	5.6	5.2	5.8	7.6	5.8	7.0
Threonine	♂	13.4	14.0	10.2	18.0	16.6	22.6
	♀	8.6	9.2	6.6	14.0	14.0	12.8
Tryptophan	♂	9.4	10.2	15.0	11.4	12.2	14.6
	♀	6.0	6.2	7.8	7.4	7.6	7.8
Valine	♂	3.6	2.2	4.2	4.8	4.6	4.2
	♀	3.8	2.2	1.4	4.4	4.2	1.4

DISCUSSION

In absolute amount, as well as in percentage of the amount ingested, histidine stands out as the exception among the "free" essential amino acids in the urine. It accounts for half or more of the essential amino acid excretion. This result confirms those of Harvey and Horwitt ('49) and Woodson et al. ('48), who worked with human subjects. The maximum excretion of histidine in the present study occurred on the wheat gluten diet (table 2); this represents less than 1% of the total intake of all essential amino acids but is 17.9%

TABLE 3

Ingestion and percentage excretion of essential amino acids by the human male, ingesting proteins of different biological values

(Figures in parentheses are biological values)

AMINO ACID	GLUTEN (0.42)		CASEIN (0.68)		BEEF (0.67)		FRESH EGG (0.95)	
	Ingested	Excreted	Ingested	Excreted	Ingested	Excreted	Ingested	Excreted
	mg/day	*%*	*mg/day*	*%*	*mg/day*	*%*	*mg/day*	*%*
Arginine	1,230	1.51	1,060	1.49	1,990	0.69	1,950	0.82
Histidine	660	17.9	825	8.2	1,010	6.9	720	8.3
Isoleucine	1,520	0.15	1,720	0.10	1,600	0.14	1,930	0.16
Leucine	2,400	0.32	2,720	0.27	2,420	0.45	2,820	0.28
Lysine	675	0.98	2,200	0.48	2,620	0.44	1,910	0.43
Methionine	560	0.11	885	0.10	843	0.06	1,010	0.06
Phenylalanine	1,680	0.63	1,470	0.76	1,210	0.79	1,770	0.63
Threonine	915	1.11	1,220	1.48	1,380	1.20	1,550	1.45
Tryptophan	304	4.93	290	3.93	312	3.91	430	3.40
Valine	1,420	0.30	1,990	0.24	1,590	0.29	2,080	0.20

of the amount of histidine ingested (table 3). With the ingestion of the other test proteins the percentage excretion of histidine was approximately half as great. The total excretion of the remaining 9 essential amino acids ranged from 0.55% to 0.67% of the total quantities ingested. With two exceptions very little correlation is apparent between the excretion of these amino acids and the biological value of the proteins. The excretion of histidine was significantly greater when the diet contained gluten than when it contained whole fresh egg as a source of protein ($P < 0.05$). On the biological value scale gluten is obviously inferior to egg protein, and hence the difference in excretion of histidine may be considered another indication of poor utilization of gluten. In considering threonine, however, the situation is reversed, and the excretion of this essential amino acid was significantly greater on the diet which contained egg protein ($P < 0.01$). In view of such inconsistencies, it is hazardous at present to generalize further on the meaning of these results.

The average daily nitrogen balances for all subjects on the various diets were: Non-protein, — 2.40 gm; casein, + 0.36 gm; non-protein, — 2.68 gm; fresh egg, + 1.04; wheat gluten, — 0.56; dried beef, + 0.33. For all periods, except when wheat gluten was fed, the total excretion of essential amino acids ranged from 141 to 152 mg/day for the male subjects and from 112 to 142 mg/day for the females. Evidently moderate deviations in either direction from nitrogen equilibrium do not affect the total urinary excretion of the essential amino acids.

The total amino acid excretion on the protein-free diet was scarcely changed by the ingestion of approximately 5 gm of protein nitrogen. The average excretion of all essential amino acids on the protein-free diet was 141 and 129 mg/day for the males and females, respectively. The addition of 30 to 35 gm of protein, regardless of biological value, increased the excretion to a maximum of 160 and 141 mg/day for the males and females, respectively. This represents a change in the males from 1.93 to 2.19 mg/kg/day or from 5.57 to

6.32 mg/day/kg$^{3/4}$. For the females the analogous calculations reveal a change from 1.99 to 2.19 mg/kg/day or from 5.66 to 6.19 mg/day/kg$^{3/4}$. In view of the limited accuracy of the analytical methods employed, these changes cannot be considered significant.

The kidney is evidently extremely efficient in the absorption of amino acids from the glomerular filtrate. This fact was demonstrated in the dog by a study of renal clearances of the 10 essential amino acids (Beyer et al., '46; Wright et al., '47; Russo et al., '47). It is difficult to understand why histidine, in the human, should be excreted in relatively large amounts and methionine in such infinitesimal quantities. The ratio of histidine to methionine excretion in the present study lay between 100 and 200. This and other discrepancies make it impossible to establish any simple relationship between renal clearances of the essential amino acids in the dog and their urinary excretion in man.

SUMMARY

The urinary excretion of "free" essential amino acids by male and female human subjects was determined when the diets contained no protein or small amounts of proteins of widely different biological value. No sex differences in amino acid excretion were detected.

With the possible exceptions of histidine and threonine, the pattern of amino acid excretion was unchanged and the absolute amounts very little changed by adding various proteins to a protein-free diet.

No correlation was found between the biological value of ingested protein and the total amount of essential amino acids excreted in the urine.

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